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ABSTRACT OF DISSERTATION

Aashish Joshi

The Graduate School

University of Kentucky

2009

SUBSTRATE AND REGULATION OF MITOCHONDRIAL μ -CALPAIN

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

By
Aashish Joshi

Lexington, Kentucky

Director: Dr. James W. Geddes, Professor of Anatomy and Neurobiology

Lexington, Kentucky

2009

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ABSTRACT OF DISSERTATION

SUBSTRATE AND REGULATION OF MITOCHONDRIAL μ -CALPAIN

μ -Calpain is localized to the mitochondrial intermembrane space. Apoptosis-inducing factor (AIF), which executes caspase-independent cell death, is also localized to the mitochondrial intermembrane space. Following processing at the N-terminus, AIF becomes truncated (tAIF) and is released from mitochondria. The protease responsible for AIF processing has not been established. The same submitochondrial localization of mitochondrial μ -calpain and AIF gives support to the hypothesis that mitochondrial μ -calpain may be responsible for processing AIF. Atractyloside-induced tAIF release in rat liver mitochondria was inhibited by cysteine protease inhibitor MDL28170, but not by calpain inhibitors PD150606 or calpastatin. Moreover, μ -calpain immunoreactivity was difficult to detect in rat liver mitochondria. In a mitochondrial fraction from SH-SY5Y cells, incubation with 5 mM Ca^{2+} resulted in the activation of mitochondrial μ -calpain but not in AIF truncation. Finally, in hippocampal neurons calpain activation did not induce AIF processing or nuclear translocation and AIF translocation to nucleus was calpain independent. The localization of μ -calpain to the mitochondrial intermembrane space is suggestive of its possible involvement in AIF processing, but direct experimental evidence supporting such a role has been elusive.

We observed that mitochondrial μ -calpain required high Ca^{2+} for activation. We examined the hypothesis that the endogenous calpain inhibitor, calpastatin, may be present in the neuronal mitochondria. Calpastatin was detected in the mitochondria-enriched fraction obtained from rat cerebral cortex and SH-SY5Y cells. The mitochondrial calpastatin was resistant to proteinase K digestion, indicating localization internal to the outer mitochondrial membrane. Submitochondrial fractionation revealed that the calpastatin was localized to the mitochondrial intermembrane space and mitoplasts (inner mitochondrial membrane and matrix) but not to the mitochondrial outer membrane fraction. Mitochondrial calpastatin was not detected when mitoplasts were incubated with proteinase K, suggesting that calpastatin is not present in the matrix. The N-terminus of XL domain of calpastatin, when fused to GFP and transfected to SH-SY5Y cells showed mitochondrial localization and thus confirmed the presence of a mitochondrial targeting sequence in calpastatin. Together, these results demonstrate the presence of calpastatin in the neuronal mitochondrial intermembrane space, the same submitochondrial compartment as mitochondrial μ -calpain. This finding explains the high Ca^{2+} requirements for mitochondrial μ -calpain activation.

KEYWORDS: Calpain, Mitochondria, Calpastatin, Apoptosis-inducing factor,
Caspase-independent cell death.

Aashish Joshi

September 4th 2009

SUBSTRATE AND REGULATION OF MITOCHONDRIAL μ -CALPAIN

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Aashish Joshi

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Dedicated to my family for their support and sacrifice

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Chapter one

Introduction

“A fact is a simple statement that everyone believes. It is innocent, unless found guilty. A hypothesis is a novel suggestion that no one wants to believe. It is guilty, until found effective.”- Edward Teller

A. Preface

“To be at the right place at the right time makes all the difference” - This adage underscores the importance of place in terms of deciding one’s fate. This simple fact of life can be extrapolated into biomedical sciences where location of a protein can significantly affect its function. At one place it may be harmless but at another place it may wreck havoc in cell. There are many examples for the above mentioned statement such as Bax, a member of BCL-2 family of pro-apoptotic proteins, is harmless in the cytosol but after appropriate trigger translocates from the cytosol to the mitochondria, oligomerizes and induces cell death (Roucou and Martinou, 2001), NFkB, a transcription factor that localizes to the cytosol in unstimulated cells, but the nuclear translocation of the same induces expression of several genes (Lenardo and Baltimore, 1989), or Cytochrome C (CytC) that is involved in cell survival in the mitochondrial intermembrane space (IMS), but once in cytosol leads to apoptosis (Liu et al., 1996). Thus, the subcellular localization of various proteins is indeed a very intelligent means of regulating functional consequences inside the cellular milieu. Calpains, the family of non-lysosomal neutral cysteine proteases, were thought be exclusively cytosolic (Murakami et al., 1981; Lane et al., 1992; Takeuchi et al., 1992; Molinari and Carafoli, 1997; Gil-Parrado et al., 2003; Goll et al., 2003; Wang et al., 2005). Recently certain isoforms of calpain have been reported to be present in the mitochondria (Garcia et al., 2005; Arrington et al., 2006; Ozaki et al., 2007; Badugu et al., 2008). The functional significance of calpain’s mitochondrial localization has not been fully addressed, possibilities being modulation of electron transport chain components, mitochondrial fusion and fission, mitochondrial enzymatic reactions or mitochondria mediated-cell death. Regulation of mitochondrial calpains is another domain that requires substantial investigation.

This dissertation is an effort to address the issue regarding the functional significance of μ -calpain’s mitochondrial localization and its regulation. We explored the

relationship between mitochondrial μ -calpain and caspase-independent cell death executioner protein, apoptosis-inducing factor (AIF). We also add to the knowledge of regulation of mitochondrial calpains by demonstrating the presence of calpain inhibitor, calpastatin, in the mitochondrial intermembrane space. We report, for the first time, presence of a mitochondrial targeting sequence in the N-terminus of calpastatin's XL domain, which not only suggests the possible mechanism of calpastatin import into the mitochondria but also provides a novel function to the yet uncharacterized XL domain of calpastatin.

B. CNS Injury

Central nervous system (CNS) injuries resulted in death of about 5.8 million people all over the world in 1998 (Kazanis, 2005). CNS injuries can be broadly classified as acquired and non-acquired injuries. Acquired CNS injury is the damage to the CNS postnatal. This includes traumatic CNS injuries such as traumatic spinal cord injury (tSCI) and traumatic brain injury (TBI) and non-traumatic CNS injuries such as substance abuse and infection. Non-acquired CNS injuries include neurodegenerative disorders such as Alzheimer's disease, amyotrophic lateral sclerosis, Huntington's disease, multiple sclerosis, Parkinson's disease. Traumatic CNS injuries (tSCI and TBI) have been tagged as a hidden epidemic. The annual incidence of traumatic CNS injuries in the United States alone is about 1.4 million (National center for injury prevention and control) (CDC, 2006), which represents one injury every half minute. Majority of the individuals afflicted are young healthy male adults - tSCI (males 1.5 times more than females) from age 0 to 4 and 15 to 19 and TBI (males 4 times more than females) from 18 to 35 years of age. Approximately 50,000 individuals die and about 246,000 are hospitalized (including 10,000 paralyses) due to traumatic CNS injuries (TBI and tSCI)(CDC, 2006) in the United States. Traumatic CNS injuries compromise an individual's sensory, motor, cognitive, executive, and emotional functions. They may also lead to chronic pain, spasticity, infertility, lack of independence and compromised life expectancy. Types of disability and symptoms vary greatly depending on the severity and location of injury. Traumatic CNS injuries are also associated with huge financial burden - US \$ 60 billion/year for direct and indirect cost of TBI (TBI, 2000) and \$ 9.7 billion/year for tSCI (SCI, 1998). Therefore there is immense need to accrue great efforts in this arena of biomedical sciences to uncover key players in the pathophysiology of

traumatic CNS injuries. This understanding will lead to the identification of novel targets that can be exploited for therapeutics, to help people suffering with pain and save economy from the burden of billions of dollars.

Efforts in basic sciences have delineated certain hallmark features in the pathophysiology of traumatic CNS injuries, including the revelation that the majority of traumatic CNS injuries are biphasic in nature (Weil et al., 2008). First is the primary phase of mechanical damage, which occurs due to the impact and transfer of a large amount of kinetic energy, leading to membrane disruption, hemorrhage, ischemia, and acute energy failure (Pettus et al., 1994; Bramlett and Dietrich, 2004). It is possible to prevent/reduce the primary mechanical damage by observing precautionary measures such as wearing helmets but it is practically impossible to treat the primary damage because it is instantaneous. The primary phase is followed by a secondary phase which lasts for days to weeks and thus provides an interventionally feasible window of opportunity for treatment (Kermer et al., 1999). Besides the long course, the secondary phase manifests a relatively better orchestrated cascade of pathophysiological events such as mechanically-induced neuronal depolarization, opening of voltage gated ion channels, increased neuronal excitation and subsequent excessive release of excitatory amino acid neurotransmitter - glutamate (Fonnum, 1984; Liu et al., 1991; Farooque et al., 1996; McAdoo et al., 1999). Excessive glutamate in the CNS further enhances neuronal excitability and increases intracellular Ca^{2+} concentration (Choi, 1988), leading to excitotoxicity (Olney, 1969). Excitotoxicity manifests severe intracellular derogatory changes including activation of phospholipases and protein kinases, disruption of mitochondrial calcium homeostasis and membrane potential ($\Delta\Psi$), increased reactive oxygen species (ROS) formation, calcium deregulation, and calpain activation (Lipton and Rosenberg, 1994; Rego and Oliveira, 2003; Park et al., 2004; Weil et al., 2008). All of these above mentioned events culminate in severe mitochondrial dysfunction, release of IMS proteins into cytosol, and initiation of apoptotic and necrotic pathways, resulting in massive tissue loss (Tator and Fehlings, 1991; Young, 1993).

Elevation of the intracellular calcium is one of the chief downstream events in secondary neurodegeneration (Happel et al., 1981; Moriya et al., 1994; Saatman et al., 1996b; Yamashima, 2000). One indispensable component of calcium deregulation is the overactivation of calpains, which contribute significantly to the neuropathology following traumatic CNS injury (Banik et al., 1997; Posmantur et al., 1997; Springer et al., 1997;

Zhao et al., 1998; Schumacher et al., 1999; Schumacher et al., 2000; Zhang et al., 2000). Thus calpain seems to be a rational target for therapeutics (Zhang et al., 2003; Yu and Geddes, 2007). Hence a better understanding of calpains, defining its substrates and regulation, would help immensely in addressing not only the issue of secondary neuronal damage following various CNS injuries like TBI, tSCI, ischemia, and stroke but also other forms of calpainopathies.

C. Calpain

1. Calpain - Introduction

Calpains (EC 3.4.22.17 ; Calcium-dependent protease with papain-like activity) were discovered by Guroff in 1964 (Guroff, 1964). Calpains are a family of well conserved 16 non-lysosomal, intracellular, neutral cysteine proteases (Ono et al., 1999; Huang and Wang, 2001) implicated in necrotic form of cell death in both *C. elegans* and mammals (Syntichaki et al., 2002; Pang et al., 2003). Calpains are found in all vertebrates and their homologues are found in invertebrates, plants and fungi (Czogalla and Sikorski, 2005). Calpains are classified on the basis of tissue distribution, as ubiquitous and tissue specific calpains, and on the basis of structure, as typical and atypical calpains (Sorimachi et al., 1994; Suzuki et al., 1995; Sorimachi et al., 1997a). Typical calpains are heterodimers consisting of a large catalytic subunit and a small regulatory subunit (Sorimachi and Suzuki, 2001) (Fig 1.1). The large catalytic subunit is an 80 kD peptide and has four domains (based on the resolved crystal structure of m-calpain) (Hosfield et al., 1999) (Fig 1.1). Domain I is a 18aa N-terminus that interacts with domain VI and is important for the stability of the heterodimer, Domain II has the catalytic triad of the enzyme and is divided into domain IIa containing the active site Cys and IIb containing His and Asn, Domain III is likely to be important for phospholipid and calcium binding (Beyers and Neumar, 2008) and also has a stretch of 17aa as a linker domain, and Domain IV has five EF hands for binding to calcium (Goll et al., 2003) (Fig 1.1). The small regulatory subunit is 28 kD and has two domains, namely domain V which is the N-terminal (Imajoh et al., 1986) glycine rich hydrophobic domain and domain VI which has five EF hands for binding to calcium (Ohno et al., 1986; Maki et al., 1997) (Fig 1.1).

2. Calpain - Functions

Calpains are involved in various physiological functions such as cell cycle progression (Zhang et al., 1997), proliferation (Zhang et al., 1996), differentiation (Grynspan et al., 1997a; Patel and Lane, 2000), migration (Franco et al., 2004; Satish et al., 2005; Wu et al., 2006), synaptic remodeling and long term potentiation (Tomimatsu et al., 2002), embryonic development (Arthur et al., 2000), meiosis and mitosis (Schollmeyer, 1988; Santella et al., 2000) as well as in pathological states including muscular dystrophy (Richard et al., 1995), diabetes (Horikawa et al., 2000), Alzheimer's disease (Saito et al., 1993; Grynspan et al., 1997b; Mark et al., 1997; Tsuji et al., 1998), Huntington's disease (Gafni and Ellerby, 2002; Lee et al., 2006), Parkinson's disease (Crocker et al., 2003), cataract (Nakamura et al., 2000), multiple sclerosis (Shields et al., 1999), neuronal ischemia (Saido et al., 1993b; Roberts-Lewis et al., 1994), and obsessive compulsive disorder (Mundo et al., 1997).

Calpains are also involved in the traumatic brain and spinal cord injuries (Kampf et al., 1996; Springer et al., 1997; Ray et al., 1999; Pike et al., 2001; Goll et al., 2003; Liu et al., 2004). Calpain activation peaks within 1-4 h post traumatic injury and leads to damage in grey matter neurons, white matter axons, oligodendrocytes, myelin, and induces inflammatory response (Wang and Yuen, 1994; Springer et al., 1997; Zhang et al., 2000; Zhang et al., 2003). Calpain-mediated proteolysis occurs for 24 h in grey matter (Ray et al., 1999) and up to two weeks in white matter (Zhang et al., 2000). Calpain inhibition is neuroprotective following tSCI (Iwasaki et al., 1985; Iwasaki, 1987; Banik et al., 1998; Ray et al., 2000; Ray et al., 2001b, a; Ray et al., 2003; Wingrave et al., 2003; Zhang et al., 2003; Yu and Geddes, 2007), TBI (Saatman et al., 1996b; Posmantur et al., 1997), and NMDA mediated excitotoxicity in primary neurons (Brorson et al., 1995).

3. Calpain - Activation

Calpain activation occurs due to the calcium induced conformational change that reduces the distance between Cys-105 and His-262, Asn-286, so as to form the catalytic triad (Hosfield et al., 1999). The Cys acts as a nucleophile and attacks on the peptidyl carbonyl, forming a tetrahedral intermediate that releases amine product and acyl-enzyme complex. The acyl-enzyme complex subsequently releases carboxyl product and the intact enzyme (Storer and Menard, 1994). Calpain activation leads to the

disassociation of the heterodimer (Yoshizawa et al., 1995; Suzuki et al., 2004) as well induces its autoproteolysis (Dayton, 1982; Cong et al., 1989; Edmunds et al., 1991; Michetti et al., 1997). Autoproteolysis makes disassociation irreversible and also decreases the amount of calcium required for activation by both m and μ -calpain by $1/10^{\text{th}}$ (Edmunds et al., 1991; Goll et al., 2003). Autoproteolysis occurs at the N-terminus and removes 27aa from the 80 kD μ -calpain large catalytic subunit to make it 76 kD, removes 18aa from 80 kD m-calpain large catalytic subunit to make it 78 kD and removes 91aa from the 28 kD small regulatory subunit to make it 18 kD (Suzuki, 1990; Graham-Siegenthaler et al., 1994). There are two ways to identify intracellular calpain activation. One is by looking for signature proteolysis of a known calpain substrate, commonly used marker is 150 kD and 145 kD bands of alpha II-spectrin (Pike et al., 1998; Wang, 2000). A second method is by examining autoproteolysis of calpain large catalytic subunits, though the lack of autolysis does not mean calpain is not active (Molinari et al., 1994; Yoshizawa et al., 1995; Elce et al., 1997; Goll et al., 2003). Being a cysteine protease calpain is highly susceptible to oxidation. Oxidation of calpains does not affect its autoproteolysis capability but inhibits its activity completely (Guttmann et al., 1997).

4. Calpain – Inhibitors

The majority of calpain inhibitors are peptide analogues (Wang, 1990; Wang and Yuen, 1997) and recent ones are non-peptide calpain inhibitors (Wang et al., 1996; Donkor, 2000). Calpain inhibitors can be classified broadly as – peptidyl epoxides, peptidyl aldehydes, peptidyl alpha ketoamids, or non-peptide inhibitors (Carragher, 2006). MDL28170 used in our studies is a peptidyl aldehyde inhibitor, which are the largest group of calpain inhibitors. These are reversible inhibitors that react with the active site thiol of the calpains (Carragher, 2006). First peptidyl aldehydes were isolated from streptomyces (Saito et al., 1972) and later generation inhibitors were made by substituting the N-terminal lipophilic group and hence termed as N-protected dipeptidyl aldehydes (Yano et al., 1993) e.g. MDL28170 (Mehdi et al., 1988). MDL28170 has shown promising results in *in vivo* models of spinal cord injury, diffuse brain injury, axonal damage, cerebral ischemia and Parkinson's disease (Iwasaki et al., 1985; Iwasaki et al., 1987; Hong et al., 1994; Banik et al., 1998; Buki et al., 2003; Crocker et al., 2003; Zhang et al., 2003; Yu and Geddes, 2007; Yu et al., 2008). PD150606 used in

our studies is a non-peptide, reversible, non-competitive calpain inhibitor that binds to allosteric site of calpain and demonstrates better pharmacokinetics (Wang et al., 1996; Carragher, 2006). PD150606 is alpha mercaptoacrylic acid (Wang et al., 1996), cell permeable and extremely selective for calpains. It binds to domain VI of calpains in the same region as the subdomain C of calpastatin (Lin et al., 1997; Todd et al., 2003). PD150606 has been shown to be effective in models of cataract, cerebellar slices suffering from excitotoxicity, and hypoxic injury in renal proximal tubes (Mathur et al., 2000; Liu et al., 2001; Xu et al., 2003). Though significant progress has been made in improvising calpain inhibitors it has to be realized that calpain inhibitors have certain lacunae such as they are non-specific (Banik et al., 1997; Crocker et al., 2003), have low potency, poor aqueous solubility, short plasma half-life, and poor BBB crossing ability (Figueiredo-Pereira et al., 1994; Wang and Yuen, 1994; Chatterjee et al., 1998). Hence novel approaches are required for calpain inhibition strategies to work in the CNS injury paradigms.

5. Calpain isoforms - μ -calpain the pathologic isoform

Calpain 1,2,3,5,10 and two small regulatory subunit isoforms are reported in the brain (Ma et al., 2001; Goll et al., 2003; Konig et al., 2003; Waghray et al., 2004). Predominant typical ubiquitous isoforms present in the central nervous system are micro (μ) and milli (m) calpains (Croall and DeMartino, 1991). They have a similar small regulatory 28 kD subunit (calpain 4), similar if not identical substrate specificities *in vitro*, and are sensitive to the same synthetic calpain inhibitors (Bever and Neumar, 2008). But they have different large catalytic subunits (50% sequence homology) and require different amounts of calcium for activation *in vitro* - micromolar concentration of calcium (2 μ M to 80 μ M) for half maximal activity for μ -calpain and millimolar concentration of calcium (0.2 mM to 0.8 mM) for half maximal activity for m-calpain (Suzuki, 1991; Saido et al., 1993a; Saido et al., 1994; Sorimachi et al., 1997a).

A convincing reason for the redundancy of similar calpains in the biological system is not evident although there are some speculations in this regard. Because of the low calcium requirement for activation, μ -calpain was assumed to be the physiological isoform of calpain but based on its distribution and cleaved substrate spectrin (Siman et al., 1985; Roberts-Lewis et al., 1994) μ -calpain may be a pathological isoform. This thought is further supported by the fact that μ -calpain knockouts are viable

and show no major phenotypic changes except platelet dysfunction (Azam et al., 2001) whereas the m-calpain knockouts die at pre-embryonic stage due to failure of blastocyst to implant (Dutt et al., 2006). Moreover, μ -calpain knockout does not alter the proteolysis of majority of cytoskeleton substrates of calpain (Franco et al., 2004) and μ -calpain knockdown is protective in NMDA excitotoxicity in hippocampal slice cultures (Bednarski et al., 1995) and disassociated neuronal cultures (Cao et al., 2007). It has also been found that m-calpain is more abundant in the CNS but it is μ -calpain that is responsible for the damage after ischemia and hence responsible for neurodegeneration (Saido et al., 1993b; Roberts-Lewis et al., 1994; Bevers and Neumar, 2008). Further it is shown that μ -calpain mediates oxidant-induced hepatocyte cell death (Miyoshi et al., 1996) and ischemic hippocampal neuronal cell death (Rami et al., 2000). These set of evidence bolsters the thought that μ -calpain is a pathologic isoform.

One paradox in the field of calpains has been that *in vivo* calcium concentration never reaches up to micro or millimolar concentration, suggesting that calpain activation must have additional components which would make its activation easier (Croall and DeMartino, 1991; Goll et al., 2003; Hood et al., 2003), such as preferred subcellular localization in the proximity of calcium stores. μ -Calpain, which has evidence of being a pathologic calpain isoform, has been reported to be present in the mitochondrial intermembrane space (Beer et al., 1982; Tavares and Duque-Magalhaes, 1991; Garcia et al., 2005; Cao et al., 2007; Badugu et al., 2008). This localization of μ -calpain would make its activation faster, easier, and reliable. This location also suggests a putative role of μ -calpain in mitochondria based pathological scenarios. **Hence, there is a convincing rationale to explore both the substrates and regulation of the mitochondrial μ -calpain, which would add immensely to the knowledge of calpain's role in various diseases particularly of the mitochondrial origin.**

6. Calpain - Substrates

Calpains process their substrates at the interdomain boundaries such that it not only modulates their activity and function (Croall and DeMartino, 1991; Friedrich and Bozoky, 2005) but also acts as a cell signaling mechanism. Calpain substrates include various classes of proteins such as cell signaling molecules, membrane proteins, intracellular enzymes, and structural proteins (Lynch and Baudry, 1987; Bi et al., 1998; Potter et al., 1998; Blomgren et al., 1999; Hasselgren, 1999; Wang, 2000; Vinade et al.,

2001; Huang et al., 2004; Liu et al., 2004; Kuchay et al., 2007). Most of the calpain substrates have been identified using *in vitro* calpain cleavage assays and thus do not necessarily reflect calpain target proteins in the cell (Liu et al., 2004). Sequence analysis of various calpain substrates has not yielded a consensus sequence or a definitive cleavage site (Cuerrier et al., 2005) and it is believed that calpain recognizes the global structural elements in the intact protein (Tomba et al., 2004). Thus all of the calpain substrates are yet not fully characterized. Calpain mediated cytoskeletal damage in the CNS injury may be the prime reason for cells to die (Saatman et al., 1996a; Saatman et al., 1998; Buki et al., 1999; Hall et al., 2005) but calpain inhibition mediated neuroprotection was not in harmony with decreased cytoskeletal damage (Saatman et al., 2000; Kupina et al., 2001). This finding supports a possibility of other calpain substrate/s being mediators of cell death. Thus, the trauma-induced and calpain-mediated mechanism that contributes to the neurodegeneration is still not known. Novel calpain substrates have been identified recently such as CRMP-3 (Hou et al., 2006), Na⁺-Ca²⁺ exchanger (NCX) (Bano et al., 2005), and mitochondrial protein apoptosis-inducing factor (AIF)(Gao and Dou, 2000; Polster et al., 2005). **The unknown calpain substrate that mediates the CNS injury pathology, the evidence that μ -calpain is the pathologic isoform and is located in the mitochondrial intermembrane space, and studies demonstrating that AIF, a mitochondrial intermembrane space protein, is a μ -calpain substrate gave enough rationale to test the hypothesis that mitochondrial μ -calpain mediated AIF processing and release.**

7. Mitochondria and apoptosis-inducing factor (AIF)-Old dog new tricks

(i) Mitochondria

Mitochondria are a good example of successful symbiotic relationship between an anaerobic eukaryotic cell and an aerobic bacterium for over 800 million years (Martin and Koonin, 2006). Mitochondria are primarily involved in energy production via citric acid cycle, electron transport chain and oxidative phosphorylation and are also extensively involved in calcium homeostasis, cell death, beta-oxidation of fatty acids, urea cycle, heme synthesis, ROS production, lipid metabolism, Xenobiotic metabolism, glucose sensing/insulin regulation, interconversion of amino acids and hormone metabolism (Reichert and Neupert, 2002; Brookes et al., 2004; Hajnoczky et al., 2006). Mitochondria are tubular, branched, and highly dynamic double membranous organelles.

Their distribution, morphology, and number changes during cell growth and cytotoxic insults by constant fusion, fission, and cytoskeletal trafficking (Lee et al., 2007).

Mitochondria are comprised of an outer mitochondrial membrane (OMM), inner mitochondrial membrane (IMM), matrix, and intermembrane space (IMS). The OMM separates cytosol from the intermembrane space. OMM is permeable to small metabolites and solutes of up to 5 kD due to voltage dependent anion channel (VDAC) (Kroemer et al., 2007). Permeabilization of the OMM releases various IMS proteins such as CytC to the cytosol to induce cell death. This phenomenon is regulated by BCL-2 family of proteins (Armstrong, 2006). BCL-2 and BCL-XL prevent the permeabilization of OMM, whereas BAX, BAK, BID, BIM, BAD, BIK promote OMM permeabilization (Esques et al., 2000; Gottlieb, 2000; Daniel et al., 2003; Chen et al., 2005). The IMM is primarily made up of cardiolipin. Normally it is impermeable to most of the ions and metabolites (van Gurp et al., 2003) and thus helps in generating the proton gradient that can be used for ATP production. It has larger surface area than OMM and forms infoldings, which project into the matrix, called cristae (Rasmussen, 1995; Frey and Mannella, 2000). Cristae increase the surface area of IMM available for respiration. It harbors the protein complexes of electron transport chain for oxidative phosphorylation. The IMM is divided into the inner boundary membrane, which is in continuation with the OMM and cristae membrane that invaginates into the matrix (Reichert and Neupert, 2002). Cristae are connected to the inner boundary membrane by tubular structures of uniform diameter (28-30 nM) and varying length, called cristae junctions (Mannella et al., 1994; Perkins and Frey, 2000). These cristae junctions act as diffusion barriers for proteins and metabolites (Mannella et al., 1997). The mitochondrial matrix is the alkaline space enclosed by IMM. It contains the mtDNA, RNA, ribosomes, calcium phosphate complexes and numerous enzymes for various biochemical reactions. The IMS is the space enclosed between the outer and inner membrane of the organelle (Palade, 1952; Sjostrand, 1953). It is a small space of about few nanometers width as thick as a lipid bilayer (Frey and Mannella, 2000; Herrmann and Hell, 2005). There are various proteins present in the IMS involved in cell death and/or cell survival. CytC is involved in the transfer of electrons between complex III and complex IV (van Gurp et al., 2003) and following release from mitochondria induces apoptotic cell death (Liu et al., 1996; Li et al., 1997). Endonuclease G (EndoG) is an endonuclease that induces nucleosomal cleavage during apoptosis (Li et al., 2001). Smac (Second mitochondrial activator of caspases) or Diablo (Direct IAP binding protein of low PI) binds to the inhibitor of

apoptosis proteins following release from IMS (Du et al., 2000; Verhagen et al., 2000). Omi/HtrA2 is a mitochondrial serine protease (Li et al., 2002), upon release from mitochondria promotes cell death by binding with inhibitor of apoptosis proteins (IAP) (Hegde et al., 2002; Verhagen et al., 2002). Apoptosis-inducing factor, an oxidoreductase, projects from inner mitochondrial membrane attachment into the IMS and the protease mitochondrial μ -calpain, whose function is yet unknown, is also present in the IMS. The IMS is further divided into the external IMS - between outer and inner mitochondrial membrane and internal IMS - between the cristae membrane separated by the cristae junctions (Frey and Mannella, 2000; Frey et al., 2002; Herrmann and Hell, 2005). Modulation of cristae junction for letting proteins flow from internal IMS to external IMS and *vice versa* is essential for cell death (Scorrano et al., 2002).

Mitochondria are the key regulators of cell death (Smith et al., 2008). On the basis of morphology cell death was broadly classified as – apoptosis, mediated by mitochondrial protein CytC via caspases and necrosis, mediated by mitochondrial permeability transition pore formation (Kroemer and Reed, 2000). Recent classification of cell death takes into account not only the morphology but also the underlying biochemical mechanism and thus other atypical modalities of cell death namely autophagy, cornification, mitotic catastrophe, anoikis, paraptosis, pyroptosis, pyronecrosis, necroapoptosis and entosis have come into picture (Kroemer et al., 2009). Caspase-independent cell death (CICD) has recently been recognized as a programmed cell death pathway presenting apoptotic morphology but without the activation of caspases and phosphatidylserine externalization, relatively slower kinetics and demonstrating only peripheral nuclear condensation (Penninger and Kroemer, 2003; Ekert et al., 2004). Caspase-independent cell death is important in both physiological and pathological situations, particularly in adult neurodegeneration (Stoka et al., 2006), displays wide variety of features depending on cell type and cell death stimuli, and essentially manifest mitochondrial outer membrane permeabilization (Tait and Green, 2008). Apoptosis-inducing factor, an IMS protein, is the key protein that actively contributes to CICD. Caspase-independent cell death pathway thus originates at mitochondria and involves AIF.

(ii) Apoptosis-inducing factor

Apoptosis-inducing factor (AIF) is a 62 kD mitochondrial flavoprotein homologous to the bacterial NADH oxidoreductase (Susin et al., 1999). It is phylogenetically conserved, encoded by a gene of 16 exons located in Xq 25-26 and A6 of chromosome X in humans and mice (Susin et al., 1999). There are four additional isoforms of AIF (Fig1.2). Apoptosis-inducing factor-exB (AIF-eXB), results from alternative usage of exon 2b (Loeffler et al., 2001). Apoptosis-inducing factor-short (AIF-sh) results from an alternate transcriptional start site at intron 9 and is a cytosolic protein corresponding to the C-terminal domain of AIF (353-613 amino acids) that retains cell death inducing properties (Delettre et al., 2006b). Apoptosis-inducing factor-short 2 (AIF-sh2) contains a previously unknown exon between exons 9 and 10 of AIF. It localizes to the mitochondria, has oxidoreductase properties, but does not show nuclear translocation and DNA damage and it is absent from normal brain tissue (Delettre et al., 2006a). Apoptosis-inducing factor-short3 (AIF-sh3) is encoded by exon 1-9b with alternative splicing of exon 2 and 9. Apoptosis-inducing factor (AIF) is required for embryonic development (Joza et al., 2001) and loss of AIF causes abnormal cell death in embryos at later stage (post E9) but earlier growth is normal (Brown et al., 2006a). Harlequin mice are AIF hypomorphs, showing 80% decline in AIF protein (Klein et al., 2002).

Apoptosis-inducing factor (AIF) is synthesized as a 67 kD precursor protein in the cytosol having a dual mitochondrial localization signal (MLS) (Fig 1.3) (Susin et al., 1999; Otera et al., 2005). Following mitochondrial import its MLS is removed at aa position 53/54 and it is anchored in the mitochondrial inner membrane as a 62 kD Type I protein (mature AIF) with its N-terminus in the matrix and C-terminus in the mitochondrial IMS (Otera et al., 2005) (Fig1.3). In normal conditions mature AIF acts as an oxidoreductase protein and free radical scavenger (Klein et al., 2002; Lipton and Bossy-Wetzel, 2002), plays role in electron transport chain (ETC) (Vahsen et al., 2004; Joza et al., 2005), and maintains mitochondrial structure (Cheung et al., 2006), whereas in pathological states it gets cleaved from the inner membrane attachment by an unknown protease (Otera et al., 2005), becomes truncated AIF (tAIF-57 kD), and translocates from the mitochondria to the nucleus via an internal C-terminal NLS and leads to extensive large scale DNA fragmentation (50 kB), chromatin condensation, and CICD (Susin et al., 1999; Daugas et al., 2000a; Joza et al., 2001; Penninger and Kroemer, 2003; Cande et al., 2004; Plesnila et al., 2004) (Fig 1.3).

Apoptosis-inducing factor (AIF) mediated CICD is reported in various neurodegeneration models. Nuclear translocation of AIF is observed in the focal cerebral ischemia (Plesnila et al., 2004), in vulnerable neurons after transient cerebral ischemia, in neuronal cultures exposed to oxygen glucose deprivation (Cao et al., 2003), in the rat model of TBI, in the neuronal cultures exposed to peroxynitrite (Zhang et al., 2002), in tSCI (Yu et al., 2006a) and in a DNA damage model (Susin et al., 1999). Apoptosis-inducing factor (AIF) release from the mitochondria and its translocation to the nucleus is documented in the MPP⁺ model of Parkinson's disease (Liou et al., 2005; Wales et al., 2008) and it is a prominent pathway of cell death following NMDA exposure in primary mouse cortical neurons, occurring even before the release of Cytochrome C (Wang et al., 2004). Apoptosis-inducing factor (AIF) is the central player in mediating PARP-1 dependent cell death (Yu et al., 2002), also called Parthanatos (Harraz et al., 2008). TNF- α induced cell death of oligodendrocytes is also mediated by AIF translocation (Jurewicz et al., 2005). AIF is released into the cytosolic fractions in Fas-mediated oligodendrocyte death (Austin and Fehlings, 2008).

Inhibition of AIF translocation is a proposed mechanism for protective effects of hepatocyte growth factor (HGF) treatment against the excitotoxic cell death of hippocampal neurons (Ishihara et al., 2005). Apoptosis-inducing factor (AIF) neutralizing antibody confers protection in p53 induced neuronal death (Cregan et al., 2004). Heat shock protein-70 (HSP70) overexpression sequesters AIF and prevents neonatal hypoxic/ischemic brain damage (Matsumori et al., 2005). Apoptosis-inducing factor (AIF) hypomorphs (Hq mice) are resistant to both PAR and NMDA toxicity and neutralizing antibodies of PAR or PARG overexpression prevented the AIF translocation to nucleus and cell death (Andrabi et al., 2006; Yu et al., 2006b). Harlequin (Hq) mice are protective in both BAX-dependent and BAX-independent neuronal injuries (Cheung et al., 2005) and show neuroprotection compared to littermate wild type mice in neonatal cerebral hypoxia-ischemia (Zhu et al., 2007). All these evidence highlight the importance of AIF-mediated CICD and possible positive implication of inhibiting AIF translocation to the nucleus in neurodegeneration. Caspase-independent cell death (CICD) being mediated by AIF release and translocation makes it imperative to understand the mechanism of AIF release from the mitochondria. The localization of μ -calpain to the mitochondrial intermembrane space (Cao et al., 2007; Badugu et al., 2008), the same submitochondrial compartment as AIF, and evidence for involvement of a unknown cysteine protease in AIF processing and release (Otera et al., 2005; Yuste et al., 2005),

places great credence to the possibility of mitochondrial μ -calpain being the protease which processes AIF.

(iii) Mitochondrial permeability transition pore

Mitochondrial permeability transition (mPT) is a sudden increase in the inner mitochondrial membrane permeability resulting from opening of a non-specific channel permeable to molecules of up to 1500 Da (Haworth and Hunter, 1979; Szabo and Zoratti, 1992). mPTP causes dissipation of mitochondrial membrane potential, loss of mitochondrial ion homeostasis, impairment of ATP synthesis, diffusion of solutes down their concentration gradient to cytosol, massive influx of water, IMM unfolding, passive mitochondrial swelling that can be measured as decrease in light scattering in suspended mitochondria (Halestrap, 1999), calcium efflux to cytosol, OMM rupture and release of various death-effector mitochondrial proteins like Cytochrome C to the cytosol (Kim et al., 2003; Sullivan et al., 2005). mPTP can be induced by multitude of factors such as Ca^{2+} , Zn^{2+} , mitochondrial toxins, NO, peroxynitrite, 4-hydroxynonenal (Bernardi, 1996), increase in the matrix calcium concentration, $\Delta\Psi$ (Bernardi, 1992), reactive oxygen species, inorganic phosphates (Bernardi et al., 1998) matrix pH (Nicolli et al., 1993), lack of adenine nucleotides (Haworth and Hunter, 1979) or use of compounds like atractyloside (Atr) that locks adenine nucleotide translocase (ANT) in the cytoplasmic face (Petronilli et al., 1994). Inhibitors of mPTP include CsA, Mg^{+2} , ADP, and Bongkerakic acid (Bernardi, 1996). The components of mPTP are under investigation, but a general consensus is that it is composed of the outer mitochondrial membrane protein VDAC, matrix protein Cyclophilin D (CypD - a peptidyl-prolyl isomerase) and IMM protein ANT (Crompton et al., 1998; Woodfield et al., 1998; Kokoszka et al., 2004; Baines et al., 2007) with a possibility of involvement of other proteins such as peripheral benzodiazepine receptor (PBR), hexokinases and creatine kinase (Kim et al., 2003). Mitochondria buffer the increased cytosolic calcium during excitotoxicity. Mitochondrial accumulation of calcium beyond the threshold results in the migration of CypD from matrix to bind to ANT and induces a conformational change in ANT to facilitate the mPTP formation. mPTP is also known to act in a low conductance mode that provides transitory openings and allows solutes of less than 343 Da to leak out and thus helps in signal transduction (Ichas and Mazat, 1998). This is the phenomenon of flickering, causing transient depolarization and calcium loss followed by restoration of $\Delta\Psi$ and

internal calcium (Ichas et al., 1997; Kerr et al., 1999; De Giorgi et al., 2000; Kindler et al., 2003; Gerencser and Adam-Vizi, 2005).

Cyclophilin-D (CypD) knockout mice show neuroprotection against CICD but not against apoptosis (Baines et al., 2005; Basso et al., 2005; Nakagawa et al., 2005). Cyclosporine A (CsA), an mPTP inhibitor (Fournier et al., 1987; Crompton et al., 1988; Broekemeier et al., 1989) which targets CypD, inhibits AIF translocation to the nucleus in transient hypoglycemic neurons (Ferrand-Drake et al., 2003) and release of tAIF (Petit et al., 1998) in the rat liver isolated mitochondria (Polster et al., 2005). These observations demonstrate the dependence of CICD i.e. AIF processing and release on mPTP. Mitochondrial permeability transition pore (mPTP) leads to huge calcium efflux and high calcium concentration in the IMS, where mitochondrial μ -calpain resides. Cyclosporine-A (CsA) also prevented calpain activation in hypoglycemic neurons (Ferrand-Drake et al., 2003), suggesting calpain activation to be downstream of mPTP. Thus it is highly likely that mitochondrial μ -calpain could be the protease that plays a central role in CICD by processing AIF following mPTP formation. **The central hypothesis tested in the chapter 3 of the dissertation is that following mitochondrial permeability transition pore formation (mPTP), mitochondrial μ -calpain is involved in the AIF processing and release.** Our results demonstrated that mitochondrial μ -calpain is not involved in the processing and release of AIF.

Studies performed in the isolated mitochondria in chapter 3 provided valuable information about the mitochondrial μ -calpain and AIF relationship in *in vitro* model. But in a cell culture model additional factors may modulate the type of relationship which exists between AIF and mitochondrial μ -calpain. Various studies performed in cell culture models have attributed different proteases in AIF processing and release, including calpain (Takano et al., 2005; Artus et al., 2006; Wales et al., 2008; Mader et al., 2009), mitochondrial calpain (Norberg et al., 2008) and μ -calpain (Cao et al., 2007; Ozaki et al., 2008). There are number of studies which suggest that outer mitochondrial membrane permeabilization is an upstream event for AIF processing and release (Tait and Green, 2008). AIF translocates to the nucleus on induction of apoptosis and necrosis and this can be prevented by BCL-2 overexpression or BAX knockouts (Daugas et al., 2000b; Cregan et al., 2002). Following glutamate stimulation of HT-22 neurons tBid translocates to the mitochondria, there is perinuclear mitochondrial accumulation, loss of mitochondrial membrane potential and Bid inhibitor and Bid siRNA

prevented the AIF release and translocation (Landshamer et al., 2008). All these are indicative of absolute necessity for outer mitochondrial membrane permeabilization for AIF proteolytic processing and mitochondrial release leading to subsequent AIF nuclear translocation. It also suggests that either translocation of a protease, probably calpain or cytosolic μ -calpain or some other factor to the mitochondrial intermembrane space from cytosol is required for AIF proteolytic processing and release. It is also possible that some inhibitory component of calpain from mitochondrial intermembrane space is released from mitochondria to the cytosol following OMM permeabilization and then AIF processing, release and translocation can occur (Modjtahedi et al., 2006). **Thus in chapter 4 of the dissertation we studied the effect of calpain activation on AIF processing and subcellular distribution in cell culture model following various cellular stressors.** These studies demonstrated that calpain activation does not necessarily induce AIF processing, AIF nuclear translocation can occur independent of calpain activity and AIF can translocate to the nucleus in full length mature 62 kD form without any processing.

8. Calpain – Regulation

Regulation of activity is essential for proper function. Calpain activity is also finely regulated by various mechanisms in the biological system, primary means of regulation are modulating calcium concentration and interaction with its specific endogenous inhibitors. Endogenous proteins such as alpha macroglobulin, L and H kininogens can inhibit calpains, but calpastatin is the only specific endogenous inhibitor exclusively for calpains with no other known function (Dayton et al., 1976; Croall and DeMartino, 1991; Mellgren, 1991; Brustis et al., 1994; De Jongh et al., 1994; Shea et al., 1995). Calpastatin is trypsin labile, heat resistant, and cannot cross the BBB and cell membranes (Okitani, 1976; Goll et al., 2003). Calpastatin inhibits both the activation and the catalytic activity of calpains (Cottin et al., 1981; Goll et al., 2003) in a reversible calcium dependent manner (Cottin et al., 1981; Imajoh and Suzuki, 1985; Otsuka and Goll, 1987).

Calpastatin does not bind to calcium itself but binds to calpain only in the presence of calcium (Wendt et al., 2004; Hanna et al., 2007). Calpastatin exists in a coil conformation in solution (Uemori et al., 1990; Konno et al., 1997) and has a migration pattern that is 40-50% slower than its true molecular weight on SDS-PAGE (Takano and

Murachi, 1982a; Maki et al., 1991). It is found only in vertebrate tissues, lower organisms such as *Drosophila*, *Caenorhabditis elegans* and *Saccharomyces cerevisiae* do not contain a calpastatin sequence (Goll et al., 2003). Calpastatin's molecular mass ranges from 17.5 kD to 84 kD and it has six domains (Goll et al., 2003). An amino terminal L domain that lacks the inhibitory activity (Maki et al., 1987; Aversa et al., 2001) followed by four repetitive inhibitory domains of 140 aa each (Emori et al., 1987; Suzuki et al., 1987; Goll et al., 2003). Each domain has three subdomains named as A, B and C (Murachi et al., 1989) and each domain inhibits one calpain molecule (Maki et al., 1987). Subdomain B binds near the catalytic center and inhibits the protease activity of calpain whereas the subdomain A and C potentiate inhibition by subdomain B. An additional N-terminal XL domain has been identified in bovines (Cong et al., 1998). Calpastatin can be cleaved by calpains due to an increase in the ratio of active calpain to calpastatin (Sorimachi et al., 1997b) and this happens in tSCI (Ray et al., 2000).

The single gene on chromosome 5 encodes calpastatin in birds and mammals (Inazawa et al., 1990; Cong et al., 1998) but there are various isoforms of calpastatin due to alternative splicing (Lee et al., 1992a; Lee et al., 1992b; Takano et al., 1999), exon skipping (De Tullio et al., 2007) and usage of different promoters (Imajoh et al., 1987; Cong et al., 1998) (Fig1.4). One alternative isoform of calpastatin in the red blood cells lacks L domain and first inhibitory domain (Takano and Murachi, 1982b). Rat brain also contains multiple mRNAs for calpastatin (De Tullio et al., 1998) and multiple isoforms in rat brain calpastatin are produced by distinct starting points and alternative splicing of the N-terminal axons (De Tullio et al., 2007). The reason for presence of so many isoforms of calpastatin is not known.

Other ways to regulate calpain activity is by translocation to the membranes, autoproteolysis, disassociation of subunits, interaction with calpain activating proteins (CAP) and phosphorylation (Croall and DeMartino, 1991; Melloni et al., 1996; Melloni et al., 1998a; Suzuki and Sorimachi, 1998; Melloni et al., 2000; Liu et al., 2004). Subcellular localization of a protein is also one of the chief mechanisms of regulating the activity by "proximity to substrates and/or activators effect". Thus for calpains, differential subcellular localization of m and μ -calpain is not only a mechanism to differentiate the function of two isoforms by limiting their substrate accessibility but also a mechanism to regulate activity by proximity to calcium store. Regulation of calpain activity can also be achieved by modulating calpastatin levels, calpastatin phosphorylation and calpastatin's

subcellular localization (Pontremoli et al., 1988; Niapour et al., 2008). XL domain has three phosphorylation sites by protein kinase A (PKA) (Murachi et al., 1989) that reduces the activity of calpastatin to inhibit calpain (Crawford et al., 1993; Melloni et al., 1996). Both protein kinase C (PKC) and PKA-mediated phosphorylation of calpastatin increases its membrane association (Adachi et al., 1991) and PKA-mediated phosphorylation helps to aggregate the molecule near the nucleus (Gil-Parrado et al., 2003) thereby differentially compartmentalizing calpastatin and calpain in the cell. Calpastatin has an amino terminal L domain having positive charged amino acids which plays a role in subcellular localization of the molecule and its regulation (Mellgren et al., 1987; Mellgren, 1988; Mellgren et al., 1989). Elevated cytosolic calcium causes dephosphorylation of calpastatin by phosphatases and promote cytosolic diffusion. Thus aggregation and subcellular distribution of calpastatin are the mechanisms of regulating calpain activity (Tullio et al., 1999).

Relatively little is known about the regulation of mitochondrial μ -calpain. Acyl-Coenzyme A Binding Protein (ACBP) has been identified as a calpain activator (Melloni et al., 2000) and has been found in the mitochondrial intermembrane space (Van Loo et al., 2002b). It has been shown further that ACBP is a necessary factor for activation of mitochondrial μ -calpain following tBid or Bid incubation (Shulga and Pastorino, 2006). In our studies we found that mitochondrial μ -calpain is hard to activate and has very high calcium requirement, one possibility could be the presence of calpastatin as a negative regulator of mitochondrial μ -calpain in the mitochondria. **Thus chapter 5 of the dissertation ventures into understanding the regulation of mitochondrial μ -calpain by probing for the presence of calpastatin in the mitochondria.** We localize calpastatin in the mitochondrial intermembrane space.

Human mitochondrial DNA is 16.6 kB circular DNA (Anderson et al., 1981). It has 37 genes and only 13 of those encode for proteins and all of them are components of electron transport chain (ETC) (Schon, 2000; Garesse and Vallejo, 2001). Majority of the mitochondrial proteins have to be synthesized in the cytosol and then imported into the mitochondria (Poyton and McEwen, 1996; Scarpulla, 2002). Matrix proteins typically have an N-terminal mitochondrial targeting sequence which forms amphipathic helix with a hydrophobic and a positively charged side (von Heijne, 1986). These are imported via interaction with translocase of outer membrane (TOM), translocase of inner membrane 23 (TIM23) and its motor in a ATP and membrane potential dependent manner

(Herrmann and Hell, 2005). Topogenesis of mitochondrial outer-membrane β -barrel proteins (TOB) complex along with TOM complex is used to integrate beta barrel proteins in the OMM. TIM 22 complexes assists in insertion of proteins in IMM, which in most cases are imported into the matrix first and then inserted into the inner mitochondrial membrane (Neupert and Herrmann, 2007). The IMS proteins can be imported by three mechanisms. Class I IMS proteins have a bipartite sequences, a mitochondrial presignal and then a hydrophobic domain. These proteins interact with TOM complex, then are pulled inside the mitochondria by TIM 23, undergo translocation arrest and finally are inserted into the IMM by lateral insertion. Presequences or the inner membrane anchors can be processed by proteases. Large proteins of IMS are usually imported by this mechanism (Herrmann and Hell, 2005). Class II IMS proteins are small in size (7-15 kD), lack N-terminal sequences and have cysteine residues that can bind to cofactors or form disulfide bridges. Unfolded proteins pass through the TOM complex and then folding traps these proteins in the IMS (Herrmann and Hell, 2005). Class III IMS proteins bind to the membrane proteins in the outer or inner mitochondrial membrane in the IMS (Herrmann and Hell, 2005). Calpastatin is nuclear encoded and has to be imported into the mitochondrial IMS. **Chapter 5 also addresses the issue regarding the mechanism of calpastatin import into the mitochondria.** Since calpastatin is a large protein localized to the mitochondrial IMS, most likely means of its import to mitochondria could be the N-terminus targeting sequence. This was confirmed by sequence analysis and further experimentation demonstrating that first 30 aa at the N-terminus of calpastatin are a mitochondrial targeting sequence.

The main goal of this dissertation was to test the possibility of AIF being a mitochondrial μ -calpain substrate and understand the regulation of mitochondrial μ -calpain. Localization of μ -calpain to the mitochondrial IMS posed a subsequent logical question regarding its function. The evidence in favor of μ -calpain's pathological role, its mitochondrial IMS localization and presence of AIF in the IMS, made AIF the most logical target of mitochondrial μ -calpain. The hypothesis tested in chapter 3 was that following mPTP formation mitochondrial μ -calpain processes and releases apoptosis-inducing factor (AIF) from mitochondrial inner membrane attachment. We report that mitochondrial μ -calpain is not involved in the processing of apoptosis-inducing factor. Chapter 4 further explored the role of calpains in AIF processing and subcellular distribution in the cell culture models. We observed calpain independent translocation of AIF to the nucleus, suggesting lack of necessity for calpains in AIF processing and

redistribution. Hence corroborating the finding in chapter 1 and suggesting that AIF processing, release and subsequent nuclear translocation could happen by more than one means. Chapter 5 of this dissertation provides evidence of mitochondrial μ -calpain regulation by establishing the localization of, calpain inhibitor, calpastatin in the mitochondrial intermembrane space. Finally this dissertation provides information regarding the mechanism of calpastatin import into the mitochondria and ascribes mitochondrial targeting sequence to the N-terminus of the XL domain of calpastatin.

Fig 1.1 Structure of calpains. A cartoon representation of the biochemical structure of calpain large catalytic subunit and calpain small regulatory subunit. The 80 kD calpain large catalytic subunit is comprised of four domains namely Domain I, II, III, and IV. Domain I is 18 amino acids long, Domain II has the catalytic triad and is further subdivided into Domain IIa, having the cysteine residue at position 105, and Domain IIb, having the histidine and asparagines residues at positions 262 and 286 respectively, Domain III has a 17 amino acids long linker domain called T domain and Domain IV has the five calcium binding EF-hands (black vertical bars). The 28 kD calpain small regulatory subunit is comprised of two domains namely Domain V and Domain VI. Domain V is glycine rich (black vertical bar) and Domain VI has the five calcium binding EF-hands (black vertical bars). Abbreviations: N-terminal-Amino terminal, C-terminal-Carboxy terminal, T-17 amino acid linker domain, aa-amino acids, Cys-Cysteine, His-Histidine, Asn-Asparagine, kD-Kilodalton.

Fig 1.1 Structure of calpains

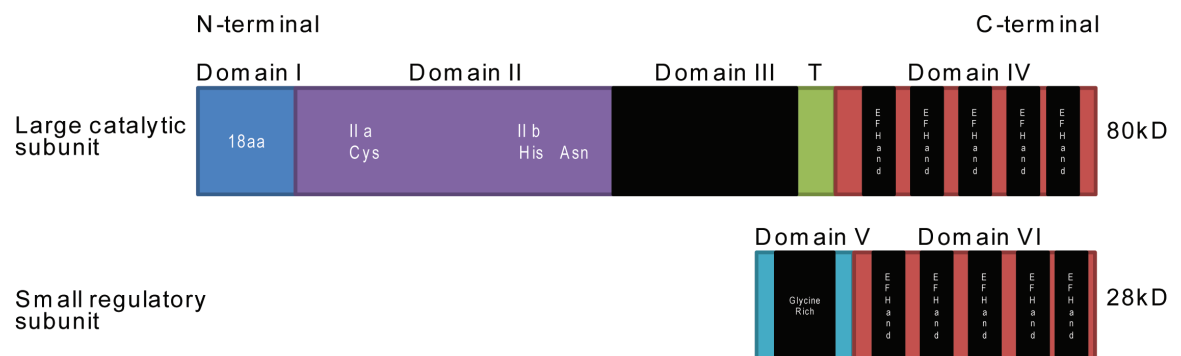


Fig 1.2 Apoptosis-inducing factor structure and isoforms. Schematic diagram of the apoptosis-inducing factor structure and isoforms, demonstrating location of oxidoreductase domain (FAD and NADH binding domains), mitochondrial localization signals, and nuclear localization signal. Apoptosis-inducing factor (AIF) contains two mitochondrial localization signals, a nuclear localization signal and an oxidoreductase domain. There are three states in which AIF can exist in a cell. Precursor AIF (67 kD) is full length, 613 amino acids long and is localized to the cytosol, mature AIF (62 kD) starts at amino acid position 55 and is anchored to the mitochondrial inner membrane and truncated AIF (tAIF-57 kD) begins at amino acid position 103 and is localized to the nucleus. There are five major isoforms of AIF. Full length AIF (Precursor AIF), encoded by exons 1-16 on X chromosome, is 613 amino acids long and is localized to the cytosol. AIF-exB, encoded by exons 1-16 but uses exon 2b instead of exon 2, is 609 amino acids long and is localized to the mitochondria. AIF-Short, encoded by exons 10-16, is 261 amino acids long and localizes to the cytosol. AIF-Short2, encoded by exons 1-9b (alternative splicing of exon 9), is 324 amino acids long and localizes to the mitochondria. AIF-Short3 is encoded by exons 1-9b (alternative splicing of exon 2 and 9) and is 237 amino acids long. The nomenclature, exon composition, and subcellular locations are listed in the periphery. The residues encompassing each domain are listed below each segment. Abbreviations: N-terminal-Amino terminal, C-terminal-Carboxy terminal, AIF-Apoptosis-inducing factor, MLS -Mitochondrial localization signal, NLS- Nuclear localization signal, FAD-Flavin adenine dinucleotide, NADH-Nicotinamide adenine dinucleotide, Met-Methionine, Ala-Alanine, Gly-Glycine, kD-Kilodalton.

Fig 1.2 Apoptosis-inducing factor structure and isoforms

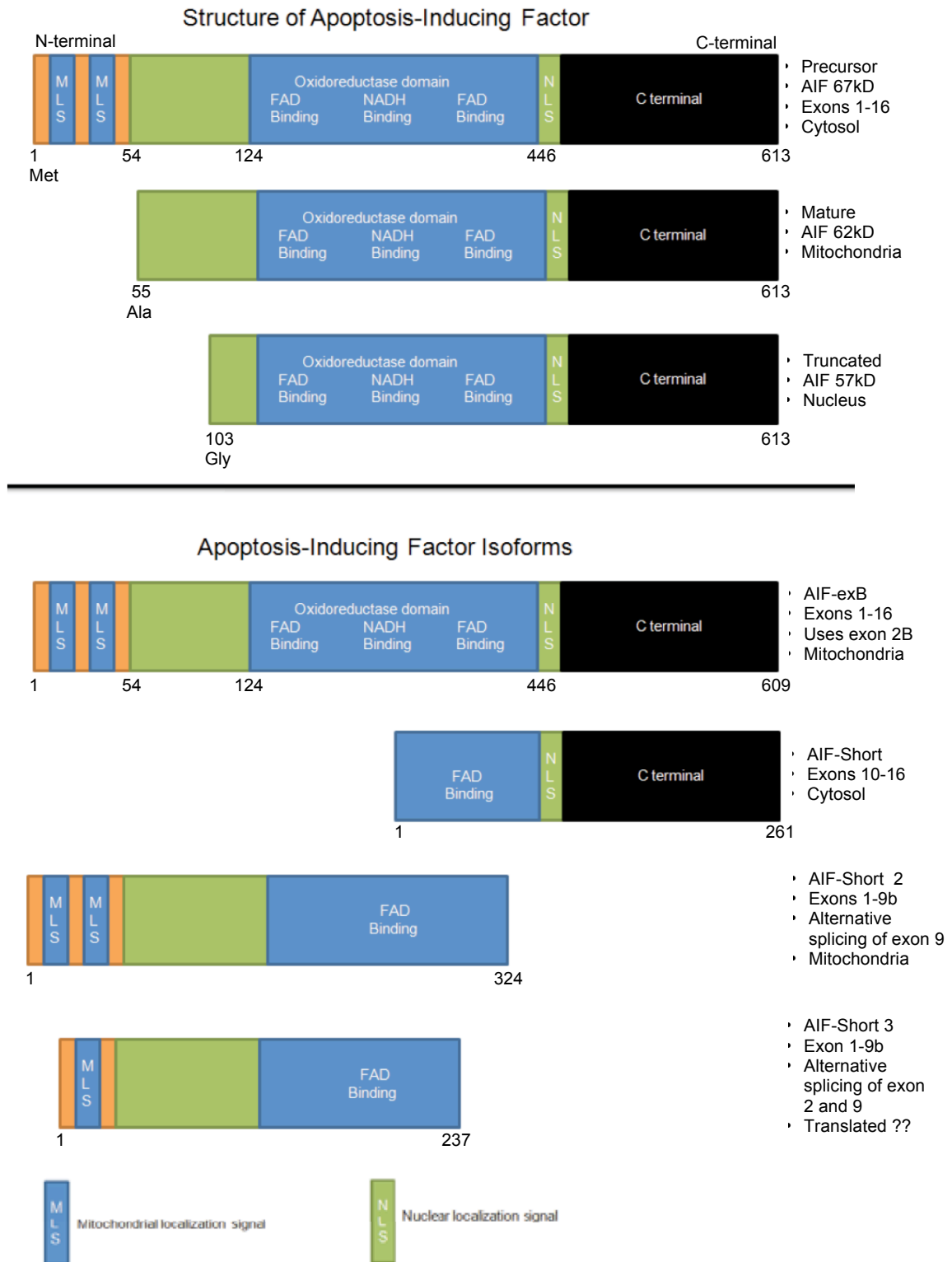


Fig 1.3 Mitochondria - Nucleus cross talk via apoptosis-inducing factor. A schematic of cross talk between mitochondria and nucleus via AIF. Precursor AIF (67 kD) after synthesis in the cytosol is imported into the mitochondria due to dual mitochondrial localization signals. Following import, mitochondrial localization signal is processed by a mitochondrial peptidase and mature AIF (62 kD) is inserted into the mitochondrial inner membrane. Mature AIF (62 kD) participates in maintaining mitochondrial structure and function. Under certain cellular death signals mature AIF is processed by an unknown protease, near its N-terminus, to be released from the inner mitochondrial membrane attachment as truncated AIF (tAIF-57 kD). This free floating tAIF, due to a nuclear localization signal, then migrates out of the mitochondria to the cytosol and finally to the nucleus. The release of tAIF is inhibited by over expression of BCL-2 and the translocation of tAIF to the nucleus is inhibited by the over expression of HSP70. Inside the nucleus tAIF induces large scale (50 kD) DNA fragmentation and caspase-independent cell death. Abbreviations: ER-Endoplasmic reticulum, SERCA-Sarcoplasmic endoplasmic reticulum Ca^{+2} -ATPase, IP3R-Inositol triphosphate receptor, AIF-Apoptosis-inducing factor, MTS-Mitochondrial targeting sequence, IMM-Inner mitochondrial membrane, OMM-Outer mitochondrial membrane, IMS-Intermembrane space, VDAC-Voltage dependent anion channel, tAIF-Truncated AIF.

Fig 1.3 Mitochondria - Nucleus cross talk via apoptosis-inducing factor

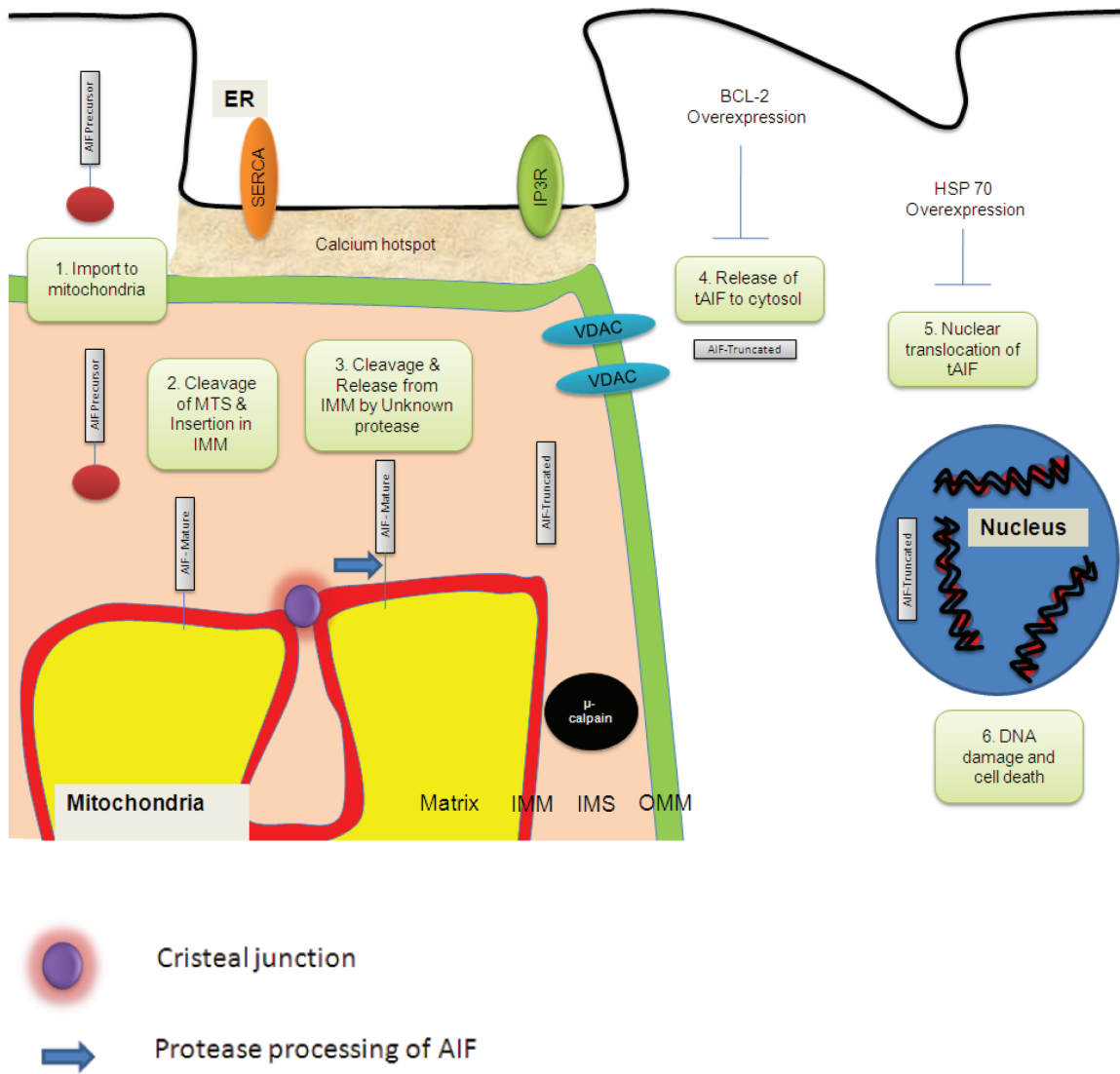
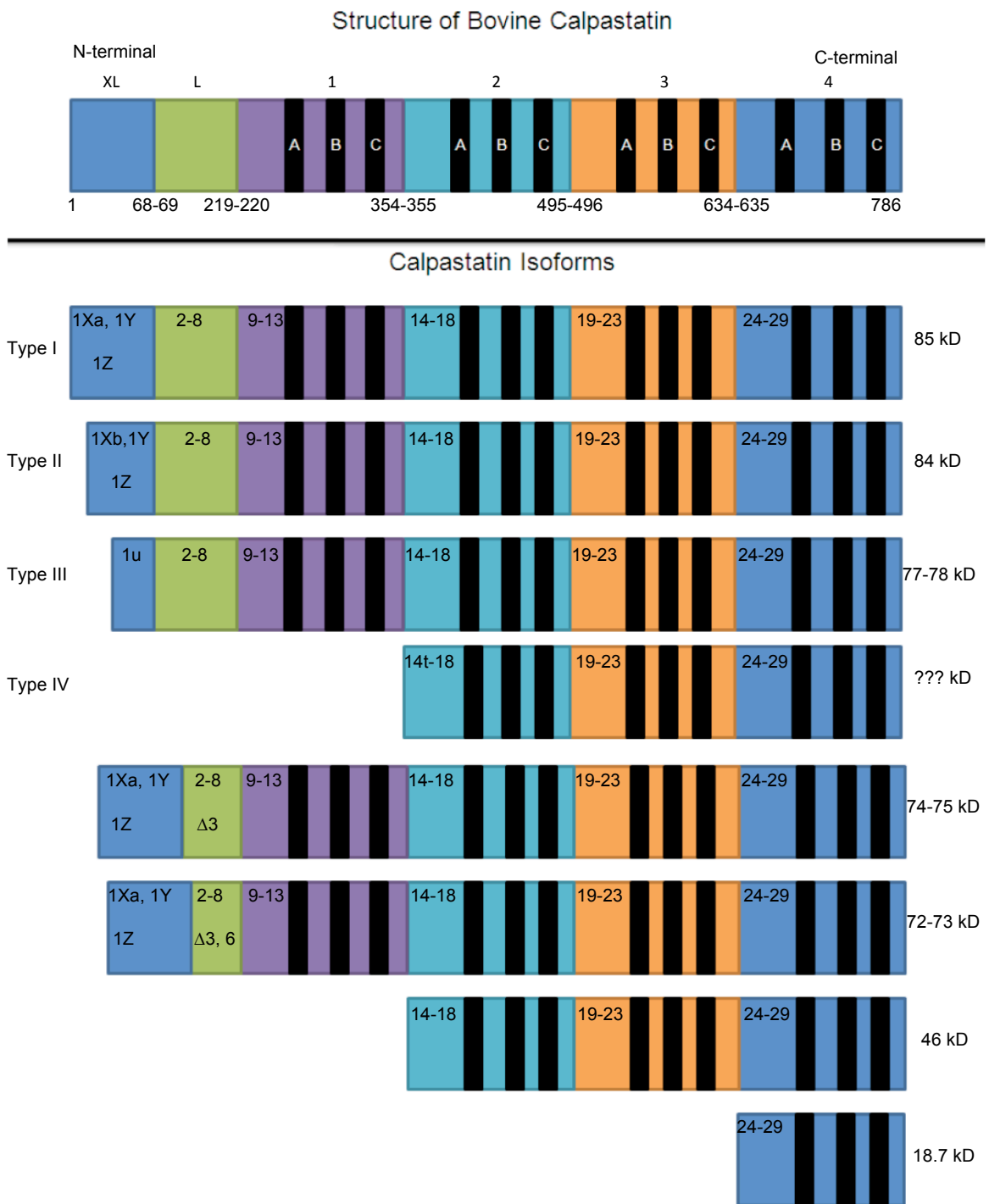


Fig 1.4 Calpastatin structure and isoforms. A representative diagram of calpastatin structure and isoforms, demonstrating the location of various domains and subdomains. Calpastatin consists of six domains - XL, L, and four inhibitory repeats. Each inhibitory repeat domain has three sub domains namely A, B, and C (black vertical bars). The residues encompassing each domain are listed below each segment. There are several isoforms of calpastatin, due to alternate splicing, exon skipping, or variable usage of promoters. The molecular weights of the various isoforms are listed in the periphery and the numbers in each segment represent the exon composition of that particular domain. Abbreviations: N-terminal-Amino terminal, C-terminal-Carboxy terminal, kD-Kilodalton.

Fig 1.4 Calpastatin structure and isoforms



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Chapter Two

Materials and Methods

“Historical methodology, as I see it, is a product of common sense applied to circumstances.” - Samuel E. Morison

Reagents used

Chemicals and reagents were obtained from Sigma Chemical (St. Louis, MO) unless otherwise noted. PD150606, CA-074 and human erythrocyte calpastatin were from Calbiochem. Potassium chloride, Glycine and Tris-base were obtained from Fisher Scientific. Acrylamide, Bis, Nitrocellulose, Molecular weight standards, Bromophenol blue and Tween-20 were from Bio-Rad. Complete EDTA free protease inhibitor cocktail tablets were purchased from Roche. Cell culture reagents including minimal essential medium (MEM), HBSS, B27, Neurobasal medium, L-glutamine, Penstrep (penicillin–streptomycin), and 0.05% Trypsin - EDTA were purchased from Invitrogen (Gibco). Fetal bovine serum was purchased from Atlanta Biologicals. Lipofectamine - 2000 was from Invitrogen. Bicinchoninic acid (BCA) kit was purchased from Pierce. Percoll was purchased through Amersham Biosciences. Alexa flour 488 secondary antibodies (cat. no. A31620 ; 1:250) for immunocytochemistry, Hoechst stain and Mitotracker red were from Molecular Probes. Calpain 1 domain III monoclonal antibody was from Calbiochem (cat. no. 208728 ; 1:2000). A polyclonal antibody against the N-terminus of calpain 1 was produced by Triple Point Biologics and purchased through Abcam (cat. no. ab28257 ; 1:2000). AIF monoclonal antibody was from Santa cruz biotechnology (E-1 clone ; cat. no. sc-13116 ; 1:1000 for western blot and 1:50 for ICC), Mitochondrial HSP70 (mHSP70) antibody was from Affinity Bioreagents (cat. no. MA3-028 ; 1:10000), Spectrin antibody was from Millipore (cat. no. MAB1622 ; 1:1000), and Calpastatin antibody was from Chemicon (cat. no. MAB3084 ; 1:1000). Secondary antibodies for western blots were IRDye 800 CW conjugated affinity purified anti-rabbit (cat. no. 611-131-122; 1:5000) or anti-mouse (cat. no. 610-131-121; 1:5000) IgG (H and L) from Rockland (Gilbertsville, PA). Rat full-length calpastatin clone was from Open Biosystems, plasmid WPXL was from Addgene. Phusion high fidelity PCR master mix with GC buffer was from New England Biolabs. QIAquick gel extraction kit and High Speed plasmid maxi

prep kit were from Qiagen. T4 ligation kit was from Invitrogen. All animals were obtained from Harlan (Indianapolis, IN).

Mitochondrial isolation

All experimental protocols involving animals were approved by the University of Kentucky Animal Use and Care Committee. Adult male Sprague–Dawley rats, weighing approximately 250 g, aged eight to twelve weeks, housed three per cage in the Division of Laboratory Animal Resources (DLAR) at the UK Medical Center accredited by AAALC, maintained on a 12 h light/12 h dark cycle were used in all experiments. All animals were fed a balanced diet *ad libitum*. The rats were anesthetized using carbon dioxide. Mitochondria were isolated from rat cerebral cortex as described previously (Brown et al., 2006b) based on method A of Sims (Sims, 1990) with slight modifications. Briefly, following CO₂ asphyxiation animals were decapitated, their brains were quickly removed and the cortices were dissected. Cortical tissue was placed in ice cold mitochondrial isolation buffer (MIB - 75 mM Sucrose, 215 mM Mannitol, 1 mM EGTA, 0.1% BSA, 20 mM HEPES, pH adjusted to 7.2 using KOH). Cortical tissues were placed into a cold glass dounce homogenizer and homogenized with six strokes. A solution consisting of equal amounts of 30% Percoll (v/v) and sample, for a final Percoll concentration of 15%, was placed on a discontinuous gradient of 24% and 40% Percoll. The density gradients were centrifuged for 10 min at 30,400 g, 4 °C in fixed angle SE-12 rotors. The fraction at 24 - 40% interface which contained the non-synaptic brain mitochondria was removed, placed into separate tubes, and diluted with MIB. The samples were then centrifuged at 16,700 g, 4 °C for 15 min. The supernatant was removed and the pellet was resuspended in MIB without EGTA and BSA. It was washed with the same buffer and centrifuged again at 11,000 g, 4 °C for 10 min. The resultant pellet was resuspended in 1 ml of MIB without EGTA and BSA, and centrifuged at 10,000 g, 4 °C for 10 min. The final mitochondrial pellets were stored on ice.

Rat liver mitochondria were isolated using combination of differential and Percoll discontinuous density gradient methods. Following CO₂ asphyxiation, the right lateral lobe of liver was dissected out, placed in MIB. Tissue was homogenized by 6 strokes at 500 rpm, centrifuged at 800 g, 4 °C for 10 min. The supernatant was centrifuged at 10,000 g, 4 °C for 10 min and the pellets resuspended in MIB. Pellets were

homogenized in the glass dounce homogenizer, and centrifuged at 3000 g, 4 °C for 5 min. The supernatant was then centrifuged at 10,000 g, 4 °C for 10 min and the pellet was resuspended in MIB, centrifuged at 9000 g, 4 °C for 10 min and resuspended the pellet in MIB. The mitochondria were then enriched using a discontinuous Percoll density gradient as described for brain mitochondrial isolation.

Mitochondria from SH-SY5Y cells were isolated as described previously (Garcia et al., 2005; Badugu et al., 2008) as per Sims method B (Sims, 1990). Briefly, SH-SY5Y cells were trypsinized and centrifuged at 2000 rpm at 4 °C for 10 min. The pellet was resuspended in MIB, incubated on ice for 10 min and then dounce homogenized by 15 strokes. A solution consisting of equal amounts of 24% Percoll (v/v) and sample, for a final Percoll concentration of 12%, was placed on a discontinuous gradient of 26% and 40% Percoll. The density gradients were centrifuged for 10 min at 30,400 g, 4 °C in fixed angle SE-12 rotors. The fraction at 26 - 40% interface which contained the mitochondria was removed, placed into separate tubes, and diluted with MIB. The samples were then centrifuged at 16,700 g, 4 °C for 15 min. The supernatant was removed and the pellet was resuspended in MIB. It was washed with the same buffer and centrifuged again at 11,000 g, 4 °C for 10 min. The resultant pellet was resuspended in 1 ml of MIB, and centrifuged at 10,000 g, 4 °C for 10 min. The final mitochondrial pellets were stored on ice.

Mitochondrial swelling assay

Mitochondrial swelling was estimated based on changes in light scattering at 540 nm, measured in a Biotek Synergy HT microplate reader (Biotek instruments, Winooski, VT). Isolated mitochondria from the rat cerebral cortex or rat liver (1 mg/ml) were resuspended in the respiration buffer (125 mM KCl, 2 mM MgCl₂, 20 mM HEPES, 2.5 mM KH₂PO₄, pH 7.2) with 5 mM pyruvate, 2.5 mM malate and 150 µM ADP. Swelling was estimated after 15 min of incubation with atractyloside (Atr) or alamethicin (Ala), or without treatment. Mitochondrial swelling is represented by decreased absorbance.

Incubation of mitochondria with atractyloside, alamethicin, and calcium

BCA protein assay was done on mitochondria from all sources. Mitochondria (1 mg/ml, protein content determined using Pierce BCA method) were resuspended in respiration buffer with pyruvate, malate, and ADP. Samples were incubated in the presence or absence of inhibitors including MDL28170 (20 μ M), PD150606 (50 μ M), human erythrocyte calpastatin (20 IU) at 4 °C for 15 min. Mitochondria were then incubated with either 5 mM atractyloside (Atr) for 30 min at 30 °C or with 15 μ M alamethicin (Alm) for 15 min at 37 °C. This was followed by centrifugation at 13,400 g for 30 min at 4 °C. The supernatants and pellets were collected, 3X sample buffer (0.5 M Tris-pH 6.8, SDS, Glycerol, β -mercaptoethanol, 1% Bromophenol blue) was added. Samples were boiled at 100 °C for 10 min and were stored at -80 °C prior to western blot analysis.

SH-SY5Y isolated mitochondria were resuspended in calpain activity buffer (20 mM Tris-HCl, 1 mM EDTA, 100 mM KCl, 0.1% β -mercaptoethanol, pH 7.5) at 2 mg/ml. Calcium chloride (0, 0.5 or 5 mM) was added and the suspension was incubated for 2 h at room temperature with shaking. Solubilized mitochondria were treated with 0.2% Triton X-100 prior to incubation with CaCl_2 . Following incubation, 3X sample buffer was added. Samples were boiled at 100 °C for 10 min and were stored at -80 °C.

Submitochondrial fractionation

Percoll-enriched mitochondria from rat brain cerebral cortex or SH-SY5Y cells were incubated in a hypotonic buffer (10 mM HEPES/KOH, pH 7.4) for 20 min on ice to rupture the outer mitochondrial membrane. 2X MIB was added after incubation to restore the isotonic conditions. It was centrifuged at 1900 g, 4 °C for 15 min on a tabletop centrifuge to obtain the mitoplast pellet. The supernatant was further centrifuged at 35,000 g (19,200 rpm in SW 55Ti rotors), 4 °C for 15 min to yield intermembrane space contents in the supernatant and outer and inner mitochondrial membrane fragments in the pellet. 3X sample buffer was added to the various fractions, boiled at 100 °C for 10 min, resolved by SDS-PAGE and analyzed by western blotting for calpastatin.

Proteinase K treatment of mitochondria and mitoplasts

Percoll-enriched mitochondria from rat brain cerebral cortex or SH-SY5Y cells or hypotonically prepared mitoplasts from SH-SY5Y cells (at 2-4 mg/ml) were resuspended in MIB, pH 8.0, and treated with proteinase K (50 µg/ml) at 37 °C for 30 min. Proteinase K activity was terminated by adding 2 mM PMSF followed by incubation on ice for 10 min. Mitochondria or mitoplasts were centrifuged at 13,000 g, 4 °C for 10 min. Pellets were resuspended in MIB and analyzed by SDS-PAGE and western blotting. Proteinase K activity was confirmed by incubating 0.2% Triton-X 100 solubilized mitochondria or mitoplasts with proteinase K (50 µg/ml), centrifuged at 13,000 g, 4 °C for 10 min and supernatants were resolved on SDS-PAGE gel and analyzed by western blotting.

Primary hippocampal cultures/ Cell culture

Primary hippocampal cultures from rats were prepared as described previously (Pang and Geddes, 1997; Sengoku et al., 2004) based on procedure of Brewer and colleagues (Brewer et al., 1993) from E18 pups, with slight modifications. The dam was euthanized by CO₂ asphyxiation, fetuses were removed aseptically, the brain was removed, and hippocampi were dissected and digested in HBSS containing 0.25% Trypsin for 15 min at room temperature. The hippocampi were washed in HBSS and incubated with Trypsin inhibitor (1 mg/ml) for 5 min. Tissue was dissociated by trituration in HBSS with a fire-polished pasteur pipette. Cells were counted using trypan blue staining and seeded at 0.3 million/35mm² into poly-D-lysine (50 µg/ml) coated glass bottom dishes containing neurobasal medium with glutamate. On next day half of the medium was replaced by neurobasal medium with B27 and glutamine. The neurons were maintained in a humidified 5% CO₂ incubator at 37 °C for 12 days. Neuronal cultures were incubated with 20 µM MDL28170 for 1 h before the treatments. NMDA was added at 100 µM in the presence of 10 µM glycine and A23187 was added at 10 µM concentration for the indicated period of times respectively.

SH-SY5Y cells were obtained from American Type Culture Collection (Manassas, VA) and propagated in MEM with non-essential amino acids, 10% fetal bovine serum, 1% penicillin–streptomycin until 80% confluent. SH-SY5Y cells were used for mitochondrial isolation or were treated with 100 µM Thapsigargin for indicated time points. Following treatments, primary hippocampal neurons or SH-SY5Y cells were either lysed in lysis buffer (62.5 mM Tris, 6M Urea, 10% Glycerol, 2% SDS, pH 6.8)

sonicated for subsequent protein assay and western blot analysis or were fixed for immunocytochemistry.

Immunocytochemistry

Immunocytochemistry was performed to evaluate AIF subcellular localization in the primary hippocampal neurons or SH-SY5Y cells. Mitochondria of these cells were labeled using mitotracker red 580 nM (1 mM). Mitotracker red was added to the culture medium for 15 min at 37 °C to label the mitochondria. Excess mitotracker was removed by washing 2X with the medium. Following treatments and mitochondrial labeling, cells were rinsed 2X in 1X PBS, fixed in 4% paraformaldehyde for 30 min, and rinsed again 3X in 1X PBS. Cells were then permeabilized in 0.2% Triton X-100 in 1X PBS for 5 min, washed 2X in 1X PBS followed by blocking in normal horse serum in 1X PBS for 30 min. Cells were washed in 1X PBS and then incubated in AIF primary antibody solution made in 1X PBS / horse serum for overnight. Next day cells were washed in 1X PBS and incubated for one hour in a species-appropriate secondary antibody conjugated with Alexa Flour 488 in 1X PBS in dark. Thereafter cells were washed in 1X PBS, followed by incubation in Hoechst 3342 (1:10,000 in 1X PBS) for 15 min to stain the nuclei. Excess stain was removed by 2X washes in 1X PBS. Finally imaging was performed on the Olympus DSU (Spinning disc confocal) IX81 motorized inverted microscope, with 2 digital cameras - EM-CCD Hamamatsu, at 100X oil objective in green, blue and red channels and Z stacks captured at a depth of 0.5 to 1.0 μ M each through the cells. This system is equipped with a 473 laser. The software used was Slidebook version 4.2 including a Deconvolution/Volume rendering module.

In situ Calpain activity assay

For *in situ* detection of calpain activity 20 μ M *t*-Boc (7-amino-4-chloromethylcoumarin, *t*-BOC-L-leucyl-L-methionine amide (CMAC, *t*-BOC-Leu-Met)) (Invitrogen) was added to the cells for 30 min. Unbound *t*-Boc was washed off before the thapsigargin treatment. Following treatment, cells were washed 2X with 1X PBS and imaging was done on fluorescent microscope at 330 nM absorbance and 403 nM emission wavelengths (Glading et al., 2000; Xu and Deng, 2006b, a).

Construction of 1-30XLDCAST-pWPXL

The N-terminus 30 amino acid sequence of the XL domain of calpastatin was PCR amplified from the rat calpastatin vector obtained from Open Biosystems - clone Id 7324026, accession no. BC 091239, catalog no. MRN1768-98079946, using forward primer 5'-ATGTCCCCGGCCCGGCCCGAA-3' and reverse primer 5'-GCATACGCGTTTCTTTTCATTAACATGC-3' having Mlu I site. Phusion high fidelity PCR mastermix with GC buffer was used for PCR reaction. Reaction conditions were – 98 °C 30s, 36 cycles at 98 °C 10s, 70 °C 10s, 72 °C 15s, and then 72 °C 5 min. PCR fragment was extracted from 2% agarose gel using QIAquick gel extraction kit, as per manufacturer's instructions. The 1-30XL domain fragment of calpastatin (1-30XLDCAST) was digested with Mlu I and pWPXL vector was digested with Pme I and Mlu I. Vector and fragment ligation was done overnight using Invitrogen T4 ligation kit. DH5 α competent cells were used for transformation and miniprep was selected using Nhe I digestion. Plasmid DNA of 1-30XLDCAST-pWPXL was extracted using QIAquick kit. Lipofectamine - 2000 transfection reagent was used as per the manufacturer's protocol for transfecting 1 μ g of 1-30XLDCAST-pWPXL vector or pWPXL vector. SH-SY5Y cells were used for the live cell imaging for 1-30XLDCAST-pWPXL or pWPXL localization. 24 h post transfection cells were visualized on the previously mentioned microscope (Olympus DSU (Spinning disc confocal) IX81 motorized inverted microscope). After GFP imaging, mitotracker red 580 nM (1 mM) was added to the culture medium for 15 min at 37 °C to label the mitochondria. Following incubation cells were washed twice with the media and imaged as mentioned above for both GFP and mitotracker staining.

Western blotting

Equal amount of protein, along with molecular weight markers, were separated by SDS-PAGE on 10% or 6.5% Tris–glycine polyacrylamide gels. The proteins were transferred to 0.45 μ M nitrocellulose membranes (Bio-Rad) by electrophoresis, and membranes were incubated in 5% nonfat dry milk powder in TBS (50 mM Tris, NaCl 0.14 M, pH7.5). Membranes were incubated with primary antibodies overnight in 5% nonfat milk and TTBS (TBS, 0.05% tween-20, pH 7.5). After three washes for 20 min each in TTBS, membranes were incubated with the species-appropriate secondary antibody for 1 h at room temperature in the dark. The membranes were washed 3X in TTBS, scanned using a Li-Cor Biosciences Odyssey infrared imaging system (Lincoln,

Nebraska, USA) and band intensity was quantified using Odyssey infrared imaging system application software version 1.2.

Statistics

Statistical calculations were performed using Statview software. Analysis consisted of Student's unpaired t-test or ANOVA followed by Fisher's LSD post hoc test for multiple comparisons. Data is expressed as mean \pm S.E.M. from at least three independent experiments. P of <0.05 was considered significant.

Chapter Three

Mitochondrial μ -calpain is not involved in the processing of apoptosis-inducing factor

Aashish Joshi, Vimala Bondada, and James W. Geddes

“There is nothing like looking, if you want to find something. You certainly usually find something, if you look, but it is not always quite the something you were after.” - J.R.R. Tolkien

Introduction

Cell death mechanisms can be broadly classified as programmed and non-programmed, with programmed cell death being further subdivided into caspase-dependent and caspase-independent mechanisms (Kroemer and Martin, 2005; Boujrad et al., 2007). Caspase-independent cell death (CICD) occurs following the cleavage and release of apoptosis-inducing factor (AIF) from mitochondria and the subsequent translocation of AIF to the nucleus, resulting in DNA fragmentation (Cregan et al., 2002; Boujrad et al., 2007). CICD is of particular importance in adult neurodegeneration (Stoka et al., 2006). CICD and AIF translocation can be induced by DNA damage resulting in the activation of poly (ADP-ribose) polymerase-1 (PARP-1) (Wang et al., 2009a) or by excessive mitochondrial Ca^{2+} uptake, the focus of the present study.

The N-terminus of mature 62 kD AIF is anchored in the inner mitochondrial membrane, with the remainder of the protein projecting into the intermembrane space. AIF release requires proteolysis near the N-terminus to generate a 57 kD fragment (Otera et al., 2005). μ -Calpain is an attractive candidate for the protease responsible for this cleavage (Liou et al., 2005; Polster et al., 2005). μ -Calpain composed of the calpain 1 large subunit and calpain small subunit-1, cleaves the 62 kD AIF to a 57 kD fragment (Polster et al., 2005). Calpain inhibitors such as calpeptin block the release of AIF from rat and mouse liver mitochondria following opening of the mitochondrial permeability transition pore by Ca^{2+} or Atr (Polster et al., 2005; Yuste et al., 2005). One difficulty with the above hypothesis is that μ -calpain was thought to be a cytosolic enzyme, which would require permeabilization of the outer mitochondrial membrane to gain access to AIF. The recent localization of μ -calpain to the mitochondrial intermembrane space avoids this issue and provides further support for the putative role of μ -calpain in the truncation of AIF (Garcia et al., 2005; Cao et al., 2007; Badugu et al., 2008; Norberg et al., 2008).

Mitochondria have a finite capacity for Ca^{2+} uptake, which when exceeded results in the opening of a non-specific mitochondrial permeability transition pore (mPTP) in the inner mitochondrial membrane. mPTP opening allows the passage of molecules less than 1.5 kD and results in the loss of mitochondrial membrane potential, release of Ca^{2+} from the mitochondrial matrix, mitochondrial swelling, and rupture of the outer mitochondrial membrane (Bernardi and Rasola, 2007). The localization of μ -calpain to the intermembrane space positions this protease to be activated by Ca^{2+} released following mPTP opening, and in the same subcellular compartment as the C-terminal region of AIF (Fig 3.1). In this study we evaluated the hypothesis that AIF processing and release is mediated by mitochondrial μ -calpain.

Results

Atractyloside-induced AIF release from isolated liver mitochondria

Isolated rat liver mitochondria were monitored for swelling at 540 nM following treatment with atractyloside (Atr) and alamethicin (Alm). Atr is a ligand for the adenine nucleotide translocase, and promotes formation of the permeability transition pore (Vieira et al., 2000). Alm, a peptide antibiotic, forms artificial channels in biological membranes (Ritov et al., 1992; Brustovetsky et al., 2002). Both drugs induced significant mitochondrial swelling and AIF release, although AIF release was more pronounced with Atr (Fig 3.2 A, B and C). AIF released into the supernatant fraction was of a slightly lower mol. wt. than AIF in the mitochondrial pellets, consistent with the requirement for AIF truncation prior to mitochondrial release and nuclear translocation (Otera et al., 2005). Atr did not induce release of matrix protein mHSP70 in the supernatant showing that Atr induced AIF release is a specific phenomenon (Fig 3.2 D).

Atractyloside did not induce AIF release from isolated brain mitochondria

As with liver mitochondria, both Atr and Alm induced swelling in isolated non-synaptic rat brain mitochondria (Fig 3.3 A). However, in contrast to rat liver mitochondria, Atr failed to induce AIF release from the rat brain mitochondria (Fig 3.3 B). The lack of response to Atr was also observed in synaptic rat brain mitochondria (results not shown). Incubation of the rat brain mitochondria with Alm did result in release of AIF into the supernatant fraction (Fig 3.3 C), similar to results obtained with rat liver mitochondria.

Atractyloside-induced AIF release is inhibited by cysteine protease inhibitor MDL28170 and cathepsin inhibitor CA-074

Previous studies have suggested the involvement of a calpain or another cysteine protease in AIF processing and release (Polster et al., 2005; Yuste et al., 2005). Preincubation of isolated liver mitochondria with 20 μ M MDL28170 (carbobenzoxy-valylphenylalaninal; calpain inhibitor III), inhibited Atr-induced AIF release from the rat liver mitochondria (Fig 3.4). However, this drug inhibits both calpains and other cysteine

proteases including cathepsin B (Wang, 1999). We therefore examined more specific calpain inhibitors including PD150606 (Wang et al., 1996) and calpastatin (Wendt et al., 2004). Both failed to prevent AIF release from rat liver mitochondria (Fig 3.4). We also examined the effect of cathepsin inhibitor CA-074 on Atr-induced AIF release. Preincubation of the isolated liver mitochondria with CA-074 significantly reduced the Atr-induced AIF release from the rat liver mitochondria (Fig 3.5). Moreover, μ -calpain immunoreactivity was difficult to detect in the mitochondria-enriched fraction from rat liver (Fig 3.6), in contrast to mitochondrial fractions from rat cerebral cortex (Fig 3.6).

Calpain activation is not sufficient for AIF release

In mitochondria isolated from rat cerebral cortex, incubation with various levels of Ca^{2+} up to 5 mM did not result in μ -calpain activation or AIF release (results not shown). One possible explanation is that the μ -calpain may have been activated during the euthanasia, brain removal, and mitochondrial isolation procedure (Garcia et al., 2005), making subsequent activation difficult. To further investigate the possible role of mitochondrial μ -calpain in AIF release, we utilized mitochondria from SH-SY5Y neuroblastoma cells, which we previously demonstrated to contain μ -calpain, and in which the calpain 1 immunoreactivity is evident as a single 80 kD band (Garcia et al., 2005; Badugu et al., 2008). Incubation of SH-SY5Y mitochondria with 5 mM, but not 0.5 mM, Ca^{2+} resulted in a lower mol. wt. calpain-1 immunoreactive band (Fig 3.7), indicative of autoproteolysis and calpain activation (Cong et al., 1989; Li et al., 2004). Calpain autolysis was also observed using an antibody specific for the N-terminus of calpain 1, which shows a loss in immunoreactivity following μ -calpain activation (Fig 3.7). Under these conditions of calpain activation, we did not detect increased AIF proteolysis in the mitochondrial fraction (Fig 3.7). Following solubilization of the mitochondria, AIF was processed to a smaller mol. wt. fragment following incubation with 5 mM Ca^{2+} (Fig 3.7, lower band indicated by arrow). MDL28170 (20 μM) inhibited the Ca^{2+} -induced calpain activation in both intact and solubilized mitochondria, and attenuated the intensity of the lower mol. wt. AIF band following Ca^{2+} incubation in the Triton-solubilized mitochondria (Fig 3.7).

Fig 3.1 Mitochondrial permeability transition. The components and regulation of the mitochondrial permeability transition pore are not fully understood, the following is a working model. In response to elevated Ca^{2+} in the mitochondrial matrix (and promoted by oxidative stress and reduced adenine nucleotides), Cyclophilin D (CypD) induces a conformational change in adenine nucleotide translocase (ANT), resulting in the opening of a non-specific channel, the mPTP. The voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane may also contribute to pore opening. This results in a rapid efflux of Ca^{2+} and other small solutes (<1500 Da) from the matrix into the mitochondrial intermembrane space and cytosol, matrix swelling, mitochondrial depolarization, and release of death-effector proteins including apoptosis-inducing factor (AIF). μ -Calpain is also located in the mitochondrial intermembrane space where it is hypothesized to be activated following mPTP opening and to cleave AIF.

Fig 3.1 Mitochondrial permeability transition

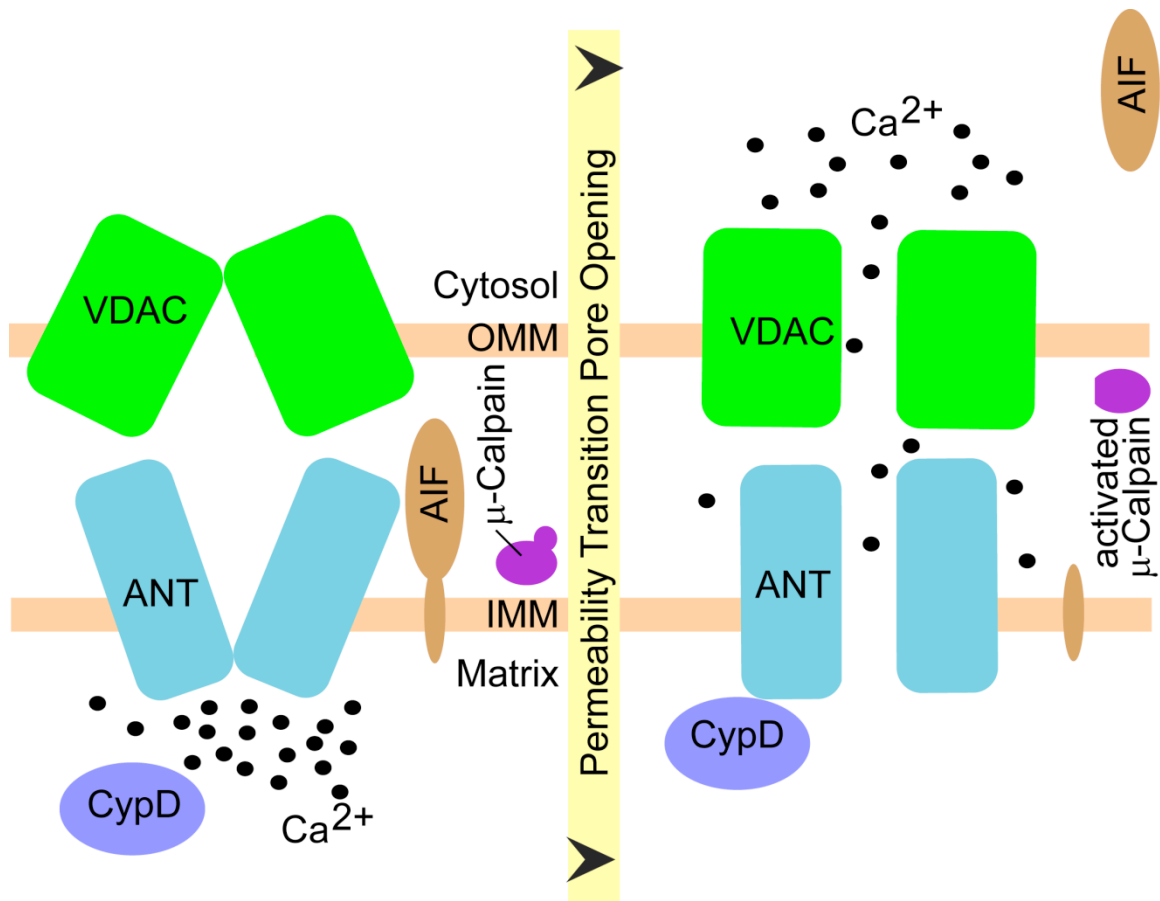


Fig 3.2 Atractyloside and Alamethicin induce AIF release from rat liver mitochondria. Isolated rat liver mitochondria were incubated with 5 mM Atr for 30 min at 30 °C or 15 µM Alm for 30 min at 37 °C in the KCl-based mitochondrial respiration buffer with pyruvate, malate and ADP. (A) The decrease in absorbance (540 nm) of the mitochondrial suspension indicates that both Atr and Alm induced swelling of the rat liver mitochondria. (B) Incubation with Atr resulted in cleavage of 62 kD AIF from the mitochondrial pellet into a 57 kD band released into the supernatant fraction. (C) Incubation of the rat liver mitochondria with Alm also resulted in the release of truncated AIF from the mitochondrial pellet into the supernatant fraction. (D) Incubation with Atr did not release matrix protein mHSP70 into the supernatant fraction. Representative western blots probed for AIF or mHSP70 are shown. The data are from at least three independent experiments, represented as mean \pm S.E.M, * indicates $P<0.05$, ** indicates $P<0.01$, NS indicates that differences were not significant at $P=0.05$ as per student's unpaired t-test or ANOVA followed by Fisher's LSD post hoc test for multiple comparisons, relative to control. Antibodies used: AIF (E-1 clone ; Santa cruz biotechnology cat. no. sc-13116 ; 1:1000), mHSP70 (Affinity Bioreagents cat. no. MA3-028 ; 1:10000). Abbreviations: Cnt-Control, Atr-Atractyloside, Alm-Alamethicin.

Fig 3.2 Atractyloside and Alamethicin induce AIF release from rat liver mitochondria.

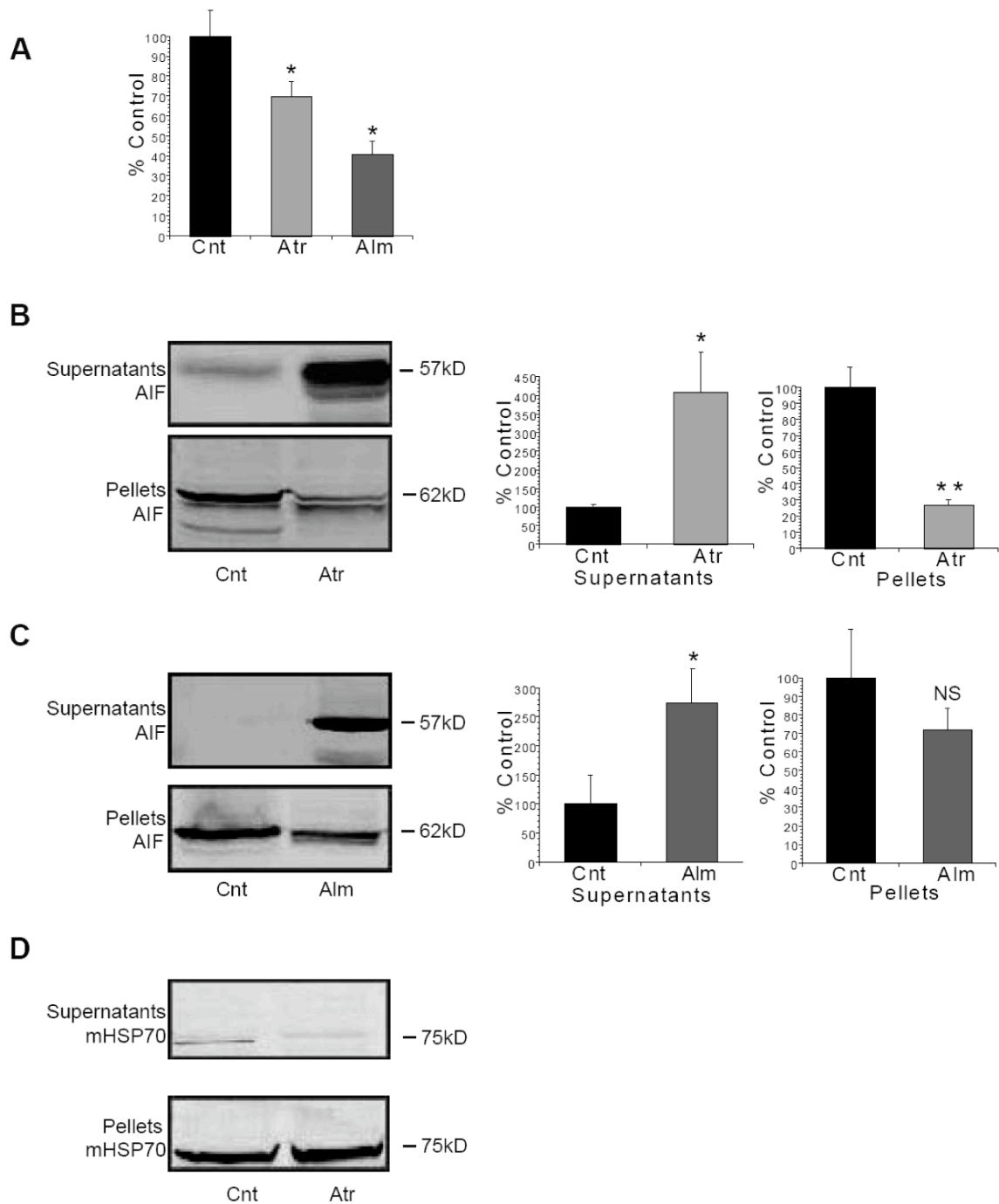


Fig 3.3 AIF is not released from rat brain mitochondria following incubation with Atractyloside. Mitochondria enriched from the cerebral cortex of the rat brain were incubated with 5 mM Atr for 30 min at 30 °C or 15 μ M Alm for 30 min at 37 °C in the KCl-based mitochondrial respiration buffer with pyruvate, malate and ADP. (A) The decrease in absorbance (540 nm) of the mitochondrial suspension indicates that both Atr and Alm induced swelling of the rat brain mitochondria. (B) Incubation with Atr did not result in the cleavage or release of AIF from the mitochondrial pellet into the supernatant fraction. (C) Incubation of the rat brain mitochondria with Alm resulted in the release of AIF from the mitochondrial pellet into the supernatant fraction. Representative western blots probed for AIF are shown. The data are from at least three independent experiments, represented as mean \pm S.E.M, * indicates $P < 0.05$, NS indicates that differences were not significant at $P = 0.05$ as per student's unpaired t-test or ANOVA followed by Fisher's LSD post hoc test for multiple comparisons, relative to control. Antibodies used: AIF (E-1 clone ; Santa cruz biotechnology cat. no. sc-13116 ; 1:1000). Abbreviations: Cnt-Control, Atr-Atractyloside, Alm-Alamethicin.

Fig 3.3 AIF is not released from rat brain mitochondria following incubation with Atractyloside.

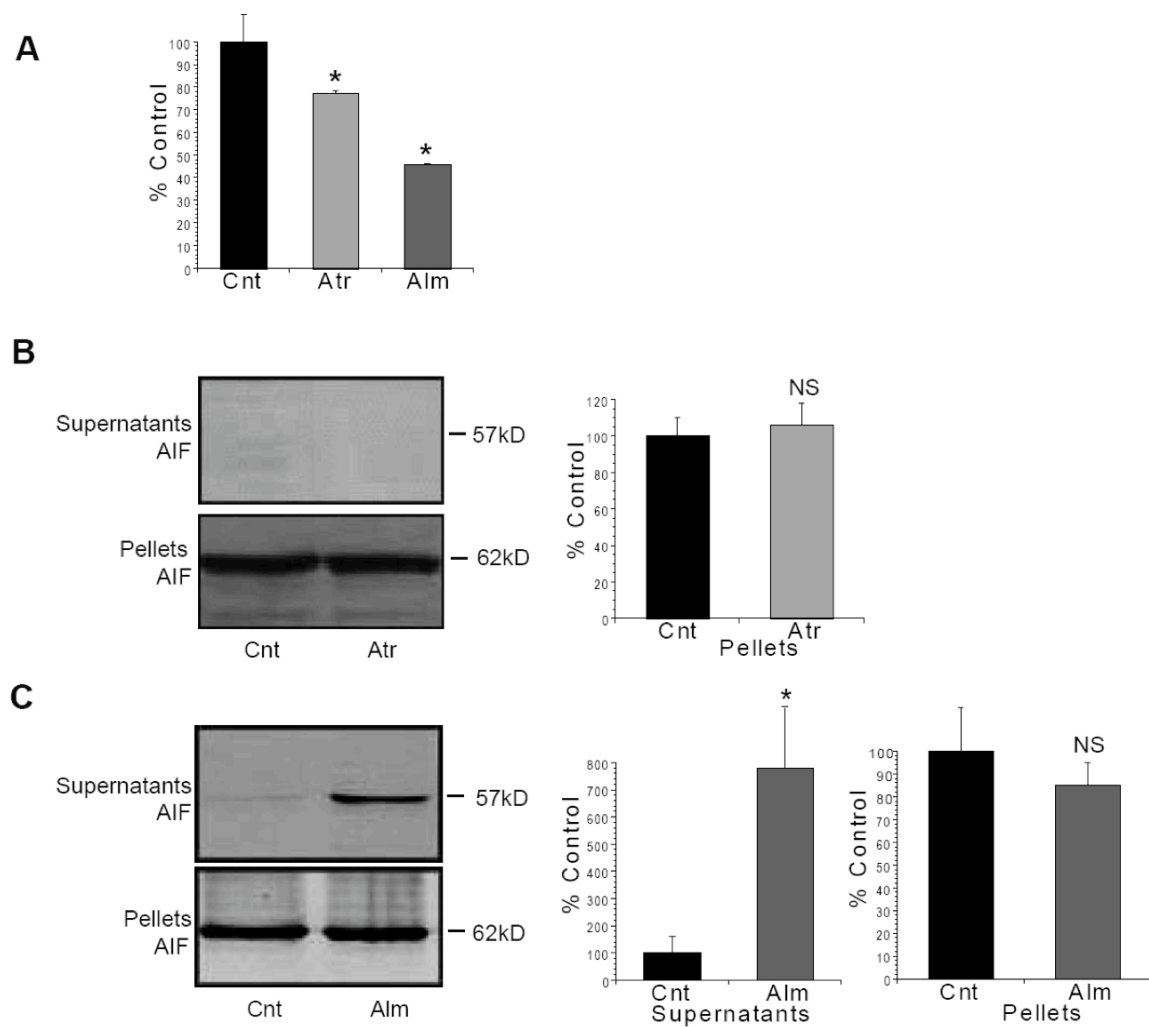


Fig 3.4 Atractyloside-induced AIF release in rat liver mitochondria was inhibited by cysteine protease inhibitor MDL28170 but not by specific calpain inhibitors PD150606 or human erythrocyte calpastatin. Isolated rat liver mitochondria were incubated with 20 μ M MDL28170 (MDL), 50 μ M PD150606 (PD), or 20 IU human erythrocyte calpastatin (CAST) at 4 °C for 15 min on ice. The mitochondria were then incubated with 5 mM Atr for 30 min at 30 °C in the KCl-based mitochondrial respiration buffer with pyruvate, malate and ADP. Supernatants (A) and pellets (B) were separated, subjected to SDS-PAGE and western blotting for AIF. MDL28170, but not PD150606 or CAST inhibited the release of AIF from the mitochondrial fraction. Representative western blots probed for AIF are shown. Data are from at least three independent experiments, represented as mean \pm S.E.M. * indicates $P < 0.05$, ** indicates $P < 0.01$, NS indicates that differences were not significant at $P = 0.05$ as per ANOVA followed by Fisher's LSD post hoc test for multiple comparisons, relative to Atr. Antibodies used: AIF (E-1 clone ; Santa cruz biotechnology cat. no. sc-13116 ; 1:1000). Abbreviations: Cnt-Control, Atr-Atractyloside, MDL-MDL28170, PD-PD150606, CAST-Calpastatin.

Fig 3.4 Atractyloside-induced AIF release in rat liver mitochondria was inhibited by cysteine protease inhibitor MDL28170 but not by specific calpain inhibitors PD150606 or human erythrocyte calpastatin.

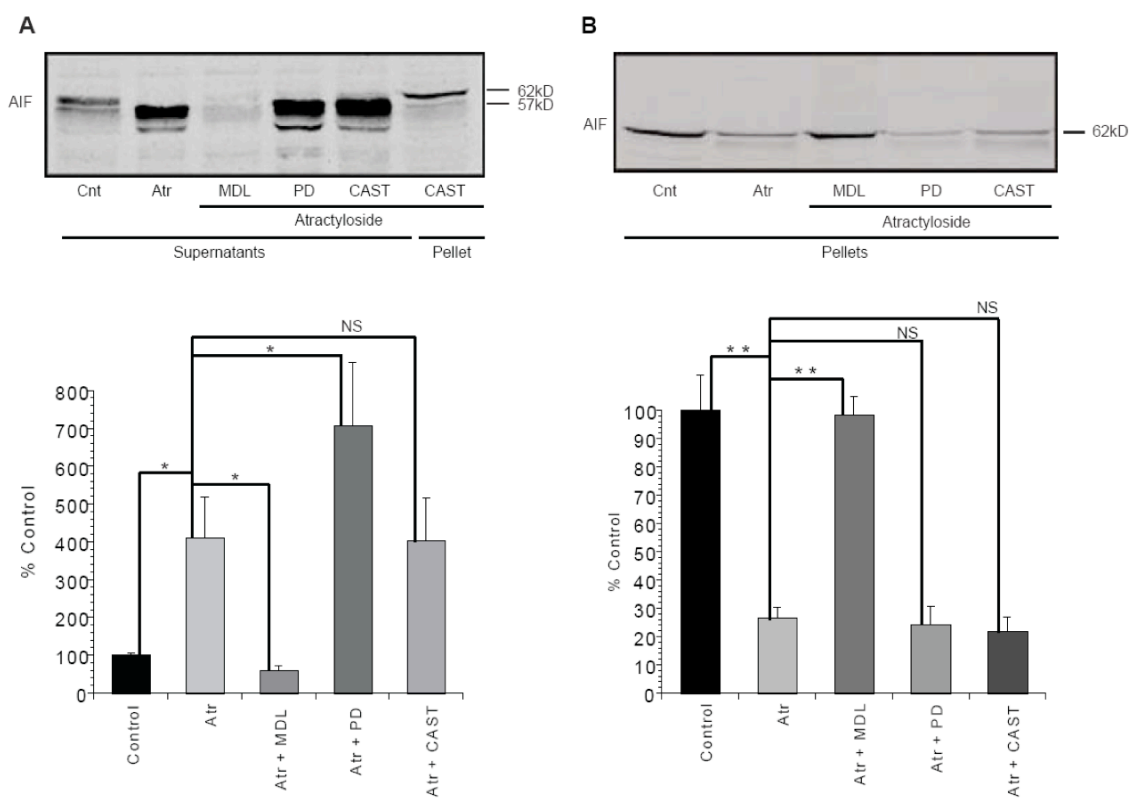


Fig 3.5 Atractyloside-induced AIF release in rat liver mitochondria was significantly reduced by cathepsin inhibitor CA-074. Isolated rat liver mitochondria were incubated with 20 μ M MDL28170 (MDL) or 20 μ M CA-074 (CA) at 4 °C for 15 min on ice. The mitochondria were then incubated with 5 mM Atr for 30 min at 30 °C in the KCl-based mitochondrial respiration buffer with pyruvate, malate and ADP. Supernatants (A) and pellets (B) were separated, subjected to SDS-PAGE and western blotting for AIF. MDL28170 completely inhibited Atr-induced AIF release and CA-074 also significantly reduced the Atr-induced release of AIF from the mitochondrial fraction. Representative western blots probed for AIF are shown. Data are from at least three independent experiments, represented as mean \pm S.E.M. * indicates $P < 0.05$ as per ANOVA followed by Fisher's LSD post hoc test for multiple comparisons, relative to Atr. Antibodies used: AIF (E-1 clone ; Santa cruz biotechnology cat. no. sc-13116 ; 1:1000). Abbreviations: Cnt-Control, Atr-Atractyloside, MDL-MDL28170, CA-CA-074.

Fig 3.5 Atractyloside-induced AIF release in rat liver mitochondria was significantly reduced by cathepsin inhibitor CA-074.

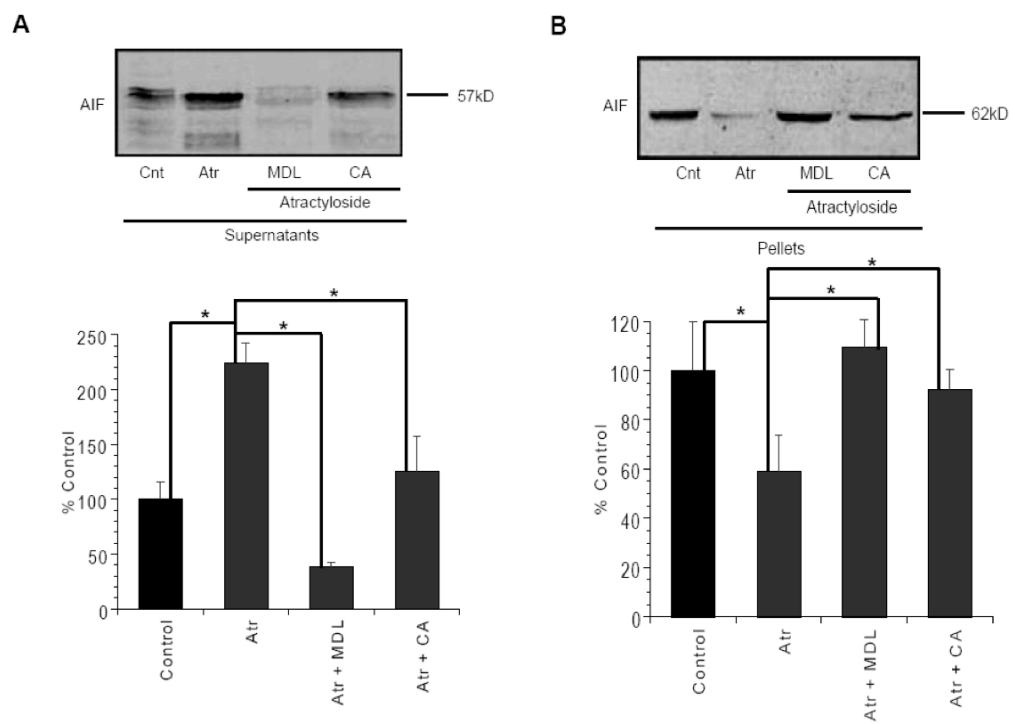


Fig 3.6 Calpain-1 immunoreactivity is difficult to detect in rat liver mitochondria. Mitochondria were isolated from rat liver and rat cerebral cortex as described in materials and methods. The starting homogenate (H) and the final mitochondrial enriched fraction (M) from the two tissues were subjected to SDS-PAGE and western blotting, and probed for calpain-1 (the large subunit of μ -calpain) using an antibody against N-terminal or for mitochondrial HSP70 (mHSP70). The results indicate an enrichment of 80 kD calpain-1 immunoreactivity in rat brain mitochondria, but very faint calpain-1 immunoreactivity in rat liver mitochondria. Representative western blots are shown. Protein loaded 80 μ g/lane. Antibodies used: Calpain-1 (Abcam cat. no. ab28257 ; 1:2000), mHSP70 (Affinity Bioreagents cat. no. MA3-028 ; 1:10000). Abbreviations: H-Homogenate, M-Mitochondrial fraction.

Fig 3.6 Calpain-1 immunoreactivity is difficult to detect in rat liver mitochondria.

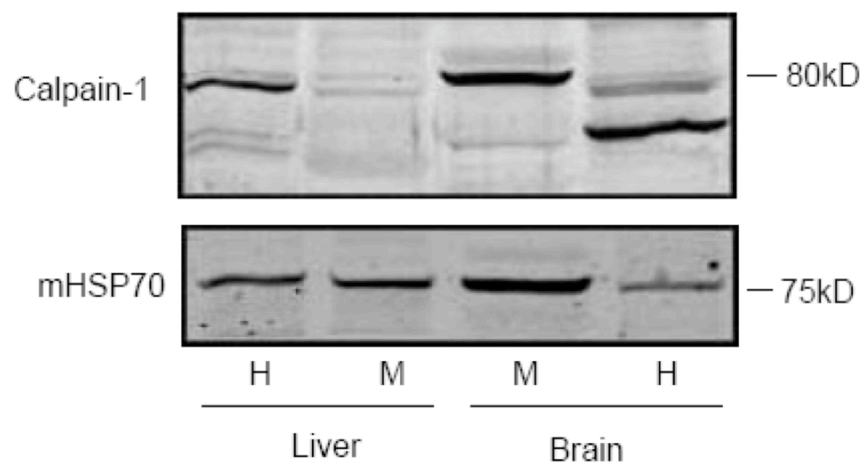
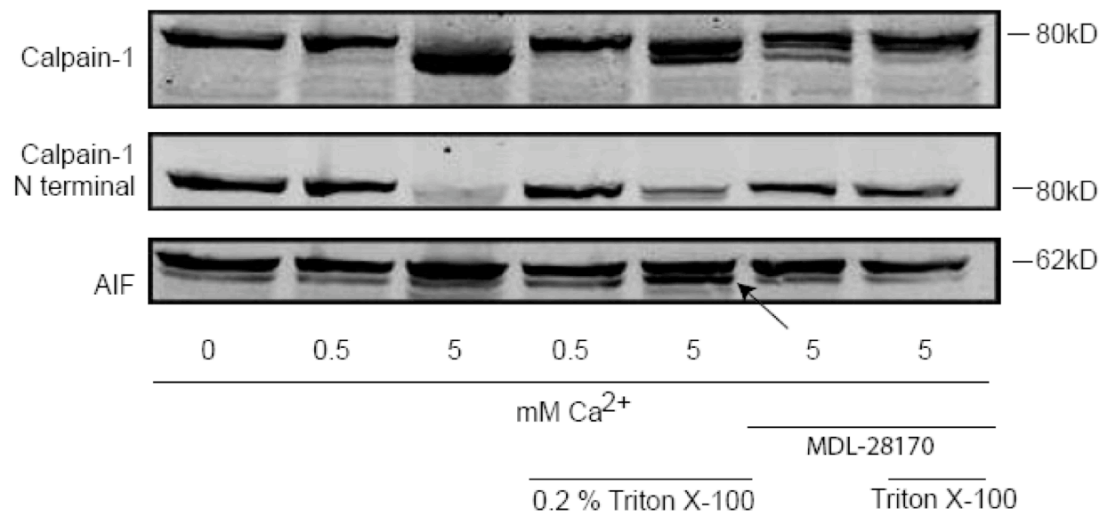


Fig 3.7 Mitochondrial μ -calpain activation does not result in AIF cleavage in SH-SY5Y mitochondria. Mitochondria enriched from SH-SY5Y cells were treated with 0.5 or 5 mM CaCl_2 , with or without Triton X-100 solubilization, and in the presence or absence of 20 μM MDL28170, in calpain activity buffer for 2 h at room temperature. Proteins were resolved by SDS-PAGE and western blotting was performed using antibodies against calpain-1 (anti-domain III and anti-N-terminus). In non-solubilized mitochondria, incubation with 5 mM, but not 0.5 mM, CaCl_2 resulted in calpain activation as indicated by autolysis of the N-terminal, resulting in a lower mol. wt. calpain-1 band and loss of N-terminal calpain-1 immunoreactivity. These conditions did not, however, result in AIF cleavage. In mitochondria solubilized with 0.2% Triton X-100, 5 mM CaCl_2 , resulted in both calpain activation and AIF cleavage. The lower mol. wt. AIF band is identified by an arrow in the lower panel. MDL28170 (20 μM) inhibited both the calpain-1 autolysis and AIF truncation induced by 5 mM Ca^{2+} . Representative western blots are shown. Antibodies used: Calpain-1 (Calbiochem cat. no. 208728 ; 1:2000), Calpain-1 N terminal (Abcam cat. no. ab28257 ; 1:2000), AIF (E-1 clone ; Santa cruz biotechnology cat. no. sc-13116 ; 1:1000).

Fig 3.7 Mitochondrial μ -calpain activation does not result in AIF cleavage in SH-SY5Y mitochondria.



Discussion

The results of this study do not support a role for mitochondrial μ -calpain in AIF release. In mitochondria enriched from rat liver, Atr-induced AIF truncation and release was inhibited by the calpain inhibitor MDL28170 but not by the more specific calpain inhibitors PD150606 and calpastatin. Although MDL28170 was designed as a calpain inhibitor and is widely used for this purpose, it also inhibits other cysteine proteases including cathepsin B (Mehdi et al., 1988; Sasaki et al., 1990; Wang, 1999). Atr-induced AIF truncation and release was significantly reduced by preincubation with cathepsin inhibitor CA-074. In addition, it was difficult to detect μ -calpain immunoreactivity in mitochondria enriched from rat liver. This contrasts with the enrichment of μ -calpain in the mitochondrial fractions vs. homogenate obtained from rat cerebral cortex (Garcia et al., 2005). Ozaki et al, (2007) were able to detect and enrich a μ -calpain like protein from swine liver. However, they used only differential centrifugation (4500 g for 10 min) to enrich mitochondria, in contrast to the combination of differential and discontinuous Percoll density gradient centrifugation utilized in the present study. It is therefore possible that at least some of the μ -calpain detected in their mitochondrial fraction represents cytosolic μ -calpain. Together, these results argue against a role for μ -calpain in the truncation and release of AIF from rat liver mitochondria following mPTP opening with Atr.

For mitochondria enriched from rat brain, we were unable to induce AIF release following incubation with either Ca^{2+} or Atr, although AIF release was observed following permeabilization with Alm. We were also unable to demonstrate μ -calpain activation under similar conditions. Several proteolytic fragments of calpain 1 are observed in mitochondria isolated from rat brain (Garcia et al., 2005), indicative of calpain activation prior to or during the isolation procedure which could interfere with the ability to activate calpain in the isolated mitochondria. We therefore examined mitochondria obtained from human SH-SY5Y neuroblastoma cells in which proteolytic calpain 1 fragments are not observed (Garcia et al., 2005). In the SH-SY5Y mitochondria, incubation with 5 mM, but not 0.5 mM, Ca^{2+} resulted in μ -calpain activation but not in AIF truncation. In Triton X-100 solubilized mitochondria, 5 mM Ca^{2+} resulted in both μ -calpain activation and AIF cleavage. The 5 mM Ca^{2+} results in Triton solubilized mitochondria are consistent with the ability of μ -calpain to cleave AIF, but suggest that AIF and μ -calpain are spatially

separated within intact mitochondria and that AIF is not a mitochondrial μ -calpain substrate.

Together, these findings argue against mitochondrial μ -calpain being the protease responsible for AIF cleavage under conditions that cause mPTP opening. In both rat brain and liver mitochondria, permeabilization of the outer mitochondrial membrane with Alm enabled AIF release. This suggests that a pool of truncated AIF is available for release following outer mitochondrial membrane permeabilization by Bid or related proteins. The requirement of outer membrane permeabilization for AIF release is consistent with several other studies (Shalbuyeva et al., 2006; Shulga and Pastorino, 2006; Landshamer et al., 2008). However, release of AIF also requires cleavage of the C-terminal 57 kD portion from the N-terminal anchor in the inner mitochondrial membrane (Otera et al., 2005). The protease responsible for AIF cleavage prior to release remains to be determined.

The ability of MDL28170, which inhibits both calpains and cysteine cathepsin proteases, to inhibit AIF release as well as significant reduction in AIF release by cathepsin inhibitor CA-074 suggests the possible involvement of a cysteine cathepsin, such as cathepsin B (Yuste et al., 2005; Chaitanya and Babu, 2008). Although cathepsin B is a lysosomal protease, an alternatively spliced variant lacking exons 2 and 3 is targeted to mitochondria (Muntener et al., 2004). However, this splice variant is catalytically inactive due to improper folding (Baici et al., 2006). In addition, cathepsin B knockout does not alter AIF release induced by a DNA alkylating agent (Moubarak et al., 2007).

Although our findings argue against the involvement of mitochondrial μ -calpain, the results do not rule out a role for cytosolic μ -calpain in AIF processing. Cytosolic μ -calpain could gain access to AIF following outer mitochondrial membrane permeabilization. In previous studies involving isolated mitochondria, exogenous μ -calpain was added to achieve mitochondrial AIF proteolysis (Liou et al., 2005; Polster et al., 2005; Cao et al., 2007). Evidence that calpastatin overexpression and calpain 1 knockdown attenuates AIF translocation following excitotoxic insult supports a role for μ -calpain, but does not distinguish between the cytosolic and mitochondrial localizations of the protease (Cao et al., 2007). In a recent study supporting a role for mitochondrial μ -calpain in AIF proteolysis, the experimental model used was digitonin permeabilized U1810 cells incubated with Ca^{2+} (Norberg et al., 2008), which would not distinguish

between the involvement of cytosolic and mitochondrial μ -calpain. Their results did demonstrate that blockade of mitochondrial Ca^{2+} uptake with ruthenium red prevented the AIF release, although this would also inhibit mPTP opening and subsequent outer mitochondrial membrane permeabilization.

In summary, the ability of μ -calpain to degrade mature AIF to a 57 kD fragment (Liou et al., 2005; Polster et al., 2005), inhibition of AIF truncation and release with specific calpain inhibitors or calpain knockdown (Cao et al., 2007; Moubarak et al., 2007; Norberg et al., 2008), and the localization of μ -calpain to the submitochondrial compartment in which AIF cleavage must occur would appear to provide compelling support for the involvement of mitochondrial μ -calpain in AIF processing. However, direct experimental support of mitochondrial μ -calpain's role in AIF truncation has been elusive.

Chapter Four

Cellular studies on the role of calpain in the processing and distribution of apoptosis-inducing factor

Aashish Joshi, Vimala Bondada, and James W. Geddes

“Nothing has such power to broaden the mind as the ability to investigate systematically and truly all that comes under thy observation in life.”- Marcus Aurelius

Introduction

Caspase-independent cell death (CICD), implicated in various diseases and disorders such as Parkinson's disease, neuronal ischemia, trauma, excitotoxicity (Cao et al., 2003; Plesnila et al., 2004; Wang et al., 2004; Liou et al., 2005; Yu et al., 2006a; Zhu et al., 2007; Wales et al., 2008), is executed by apoptosis-inducing factor (AIF) (Cregan et al., 2002; Boujrad et al., 2007). Mature AIF is a 62 kD flavoprotein, a bacterial homologue oxidoreductase (Susin et al., 1999), which is anchored to the mitochondrial inner membrane and participates in oxidative phosphorylation, mitochondrial structure maintenance and scavenging free radicals (Klein et al., 2002; Vahsen et al., 2004; Joza et al., 2005). Under various pathological conditions mature 62 kD AIF gets processed into a 57 kD tAIF (Otera et al., 2005). Processing of AIF disjuncts it from inner mitochondrial membrane attachment and exposes its nuclear localization signal (NLS). Due to NLS, tAIF translocates from mitochondria to the nucleus and induces large scale (50 kD) DNA fragmentation in conjunction with Endonuclease G (EndoG) (Susin et al., 1999; Wang et al., 2002; Wang et al., 2004; Delettre et al., 2006b; Delettre et al., 2006a).

Mature 62 kD AIF is processed at 101 amino acid position for release from inner mitochondrial membrane attachment (Otera et al., 2005). Various proteases have been implicated in this processing including caspases, cathepsins, or calpains. Calpains are non-lysosomal neutral cysteine proteases (Guroff, 1964; Goll et al., 2003). Studies have demonstrated involvement of μ -calpain in the AIF processing and subsequent nuclear translocation in MPP⁺ model of Parkinson's disease (Wales et al., 2008), LL-37 peptide induced cell death of Jurkat T cells (Mader et al., 2009), staurosporine treated U1810

cells (Norberg et al., 2008), C44 induced cell death in HEL cells (Artus et al., 2006), and cerebral ischemia (Cao et al., 2007).

We demonstrated in chapter 3 that mitochondrial μ -calpain is not involved in the processing of AIF in the isolated mitochondria (Joshi et al., 2009). Not all μ -calpain is mitochondrial, some μ -calpain is thought to be cytosolic and calpains have ability to translocate to membranes (Kuboki et al., 1992; Goll et al., 2003). Thus one possibility is that cytosolic μ -calpain, following outer mitochondrial membrane permeabilization, may get translocated to the mitochondria and induce AIF processing and subsequent nuclear translocation. The purpose of this study was to determine the role of calpain in AIF processing and subcellular distribution. We activated calpain via three distinct mechanisms by using NMDA or A23187 in the primary hippocampal neurons and Thapsigargin in the SH-SY5Y human neuroblastoma cells. We report that calpain activation does not necessarily lead to AIF processing, AIF nuclear translocation can occur independent of calpain activity and AIF can translocate to the nucleus in full length mature 62 kD form even without getting processed.

Results

AIF processing is insult dependent

Calpains can be over activated by sustained calcium overload achieved using calcium ionophore A23187 or NMDA (Prehn et al., 1994; Brorson et al., 1995; Wang, 2000). To investigate the role of calpain in AIF processing, we used NMDA or A23187 treatment model in the primary hippocampal neurons. Both drugs induced calpain activation as demonstrated by 150 kD and 145 kD band of spectrin at all time points of treatment (Fig 4.1). We did not detect lower molecular weight band of AIF (tAIF) (Fig 4.1), suggesting lack of AIF processing despite NMDA/A23187 induced calpain activation in the hippocampal cultures.

SH-SY5Y cells treated with low dose of thapsigargin (0.1 to 10 μ M) for 24 h did not demonstrate μ -calpain autoproteolysis or AIF processing (Fig 4.4 A). High dose of thapsigargin (100 μ M) induced robust calpain activation as demonstrated by μ -calpain autoproteolysis (Fig 4.4 A), presence of 145 kD spectrin breakdown band (Fig 4.4 B) and increased signal in the tBOC assay (Fig 4.4 C). It also demonstrated concomitant AIF processing visualized as a faster migrating band on the immunoblot (Fig 4.4 A and D).

AIF distribution is insult dependent

To study the effect of calpain activation on AIF subcellular localization primary hippocampal neurons were treated with NMDA or A23187 and SH-SY5Y cells were treated with Thapsigargin. Following NMDA treatment there was significant change in the cellular morphology and AIF distribution (Fig 4.2). AIF was observed to cluster around the nucleus in a perinuclear notch along with mitochondria, but no AIF immunostaining was detected in the nucleus (Fig 4.2), suggesting that under calpain activation paradigms AIF is not necessarily translocated to the nucleus. The change in cellular morphology and AIF distribution was sensitive to calpain inhibitor MDL28170 pretreatment (Fig 4.2). In contrast to NMDA, A23187 exposure induced robust nuclear translocation of AIF, which was unaffected by MDL28170 pretreatment (Fig 4.3). In the SH-SY5Y cells high dose (100 μ M) thapsigargin induced AIF distribution to the cytosol but did not demonstrate nuclear translocation of AIF (Fig 4.5).

Table 4.1 Calpain activation, AIF processing, AIF distribution and effect of MDL on AIF distribution in various treatment paradigms. Calpain activation is observed in NMDA and A23187 treatment paradigms in the hippocampal neurons and in thapsigargin treatment paradigm in the SH-SY5Y cells. AIF processing is observed in only high dose (100 μ M) thapsigargin treatment paradigm in the SH-SY5Y cells. AIF nuclear translocation is observed in the A23187 treatment paradigm in the hippocampal neurons with or without MDL28170 pretreatment. AIF cytosolic distribution is observed in the high dose (100 μ M) thapsigargin treatment paradigm in the SH-SY5Y cells. Abbreviations: NA- not applicable, W.B. – Western blotting, I.C.C. – Immunocytochemistry.

Table 4.1 Calpain activation, AIF processing, AIF distribution and effect of MDL28170 on AIF distribution in various treatment paradigms.

Technique	Parameter	NMDA	A23187	Thapsigargin
W.B. - Spectrin	Calpain activation	+	+	+
W.B. - AIF	tAIF	-	-	+
I.C.C. - AIF	AIF extra mitochondrial distribution	-	+	+
I.C.C. - AIF	Effect of MDL on AIF extra mitochondrial distribution	NA	-	NA

Fig 4.1 NMDA or A23187 induced calpain activation does not lead to AIF processing. Primary hippocampal neuronal cultures from E18 pups at 12 DIV were treated with NMDA (100 μ M) or calcium ionophore A23187 (10 μ M) for the indicated time points. Protein extracts were analyzed by SDS-PAGE and western blotting for spectrin and AIF. Increased proteolysis of spectrin, demonstrating robust calpain activation in the cultures following NMDA or A23187 exposure, was observed in both treatments at all time points. Despite calpain activation no processing of AIF was visible on the immunoblot. Representative western blots are shown. Antibodies used: Spectrin (Millipore cat. no. MAB1622 ; 1:1000), AIF (E-1 clone ; Santa cruz biotechnology cat. no. sc-13116 ; 1:1000). Abbreviations: Cnt-Control, AIF-Apoptosis-inducing factor, E18-Embryonic day 18, DIV-Days *in vitro*.

Fig 4.1 NMDA or A23187 induced calpain activation does not lead to AIF processing.

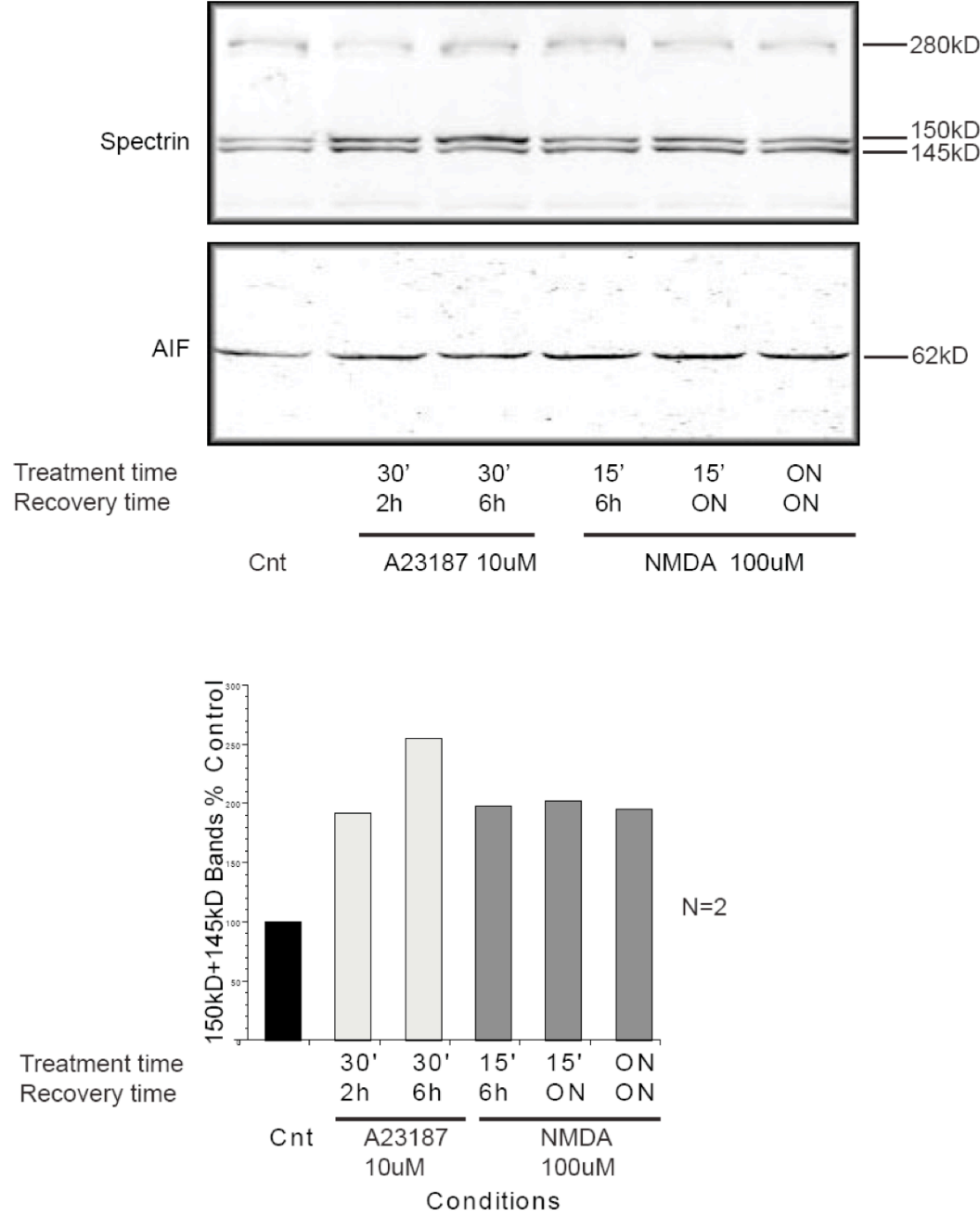


Fig 4.2 AIF attains perinuclear staining following NMDA treatment. Primary hippocampal neuronal cultures from E18 pups at 12 DIV were treated with NMDA (100 μ M) overnight in the presence or absence of MDL28170 (20 μ M). Following incubation mitochondria were stained by mitotracker red 580 nm (1 mM) (Red channel) for 15 min, then neurons were fixed, permeabilized and immunostained for AIF (Green channel), and stained by Hoechst for nucleus (Blue channel). Representative confocal photomicrographs of neurons from three independent experiments are shown. Antibodies used: AIF (E-1 clone ; Santa cruz biotechnology cat. no. sc-13116 ; 1:50). Abbreviations: MDL-MDL28170, AIF-Apoptosis-inducing factor, E-18-Embryonic day 18, DIV-Days *in vitro*.

Fig 4.2 AIF attains perinuclear staining following NMDA treatment.

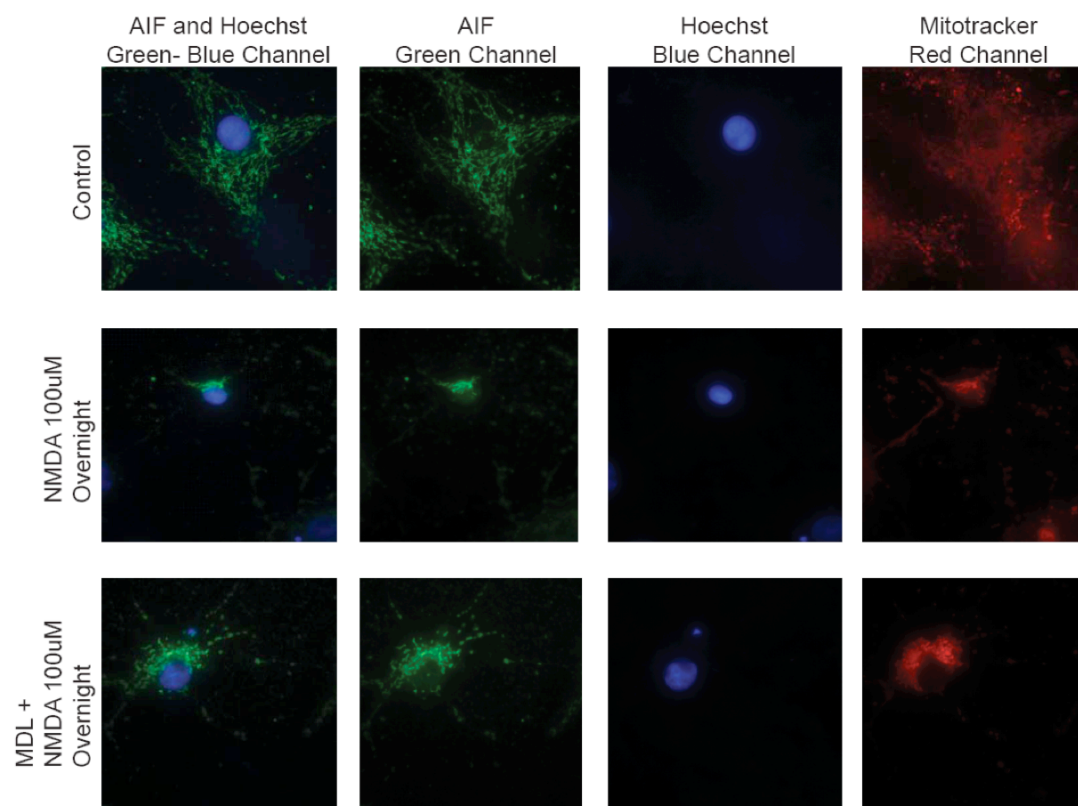


Fig 4.3 AIF translocates to the nucleus following A23187 treatment. Primary hippocampal neuronal cultures from E18 pups at 12 DIV were treated with calcium ionophore A23187 (10 μ M) for indicated time points in the presence or absence of MDL28170 (20 μ M). Following incubation mitochondria were stained by mitotracker red 580 nm (1 mM) (Red channel) for 15 min, then neurons were fixed, permeabilized and immunostained for AIF (Green channel), and stained by Hoechst for nucleus (Blue channel). Representative confocal photomicrographs of neurons from three independent experiments are shown. Antibodies used: AIF (E-1 clone ; Santa cruz biotechnology cat. no. sc-13116 ; 1:50). Abbreviations: MDL-MDL28170, AIF-Apoptosis-inducing factor, E-18-Embryonic day 18, DIV-Days *in vitro*.

Fig 4.3 AIF translocates to the nucleus following A23187 treatment.

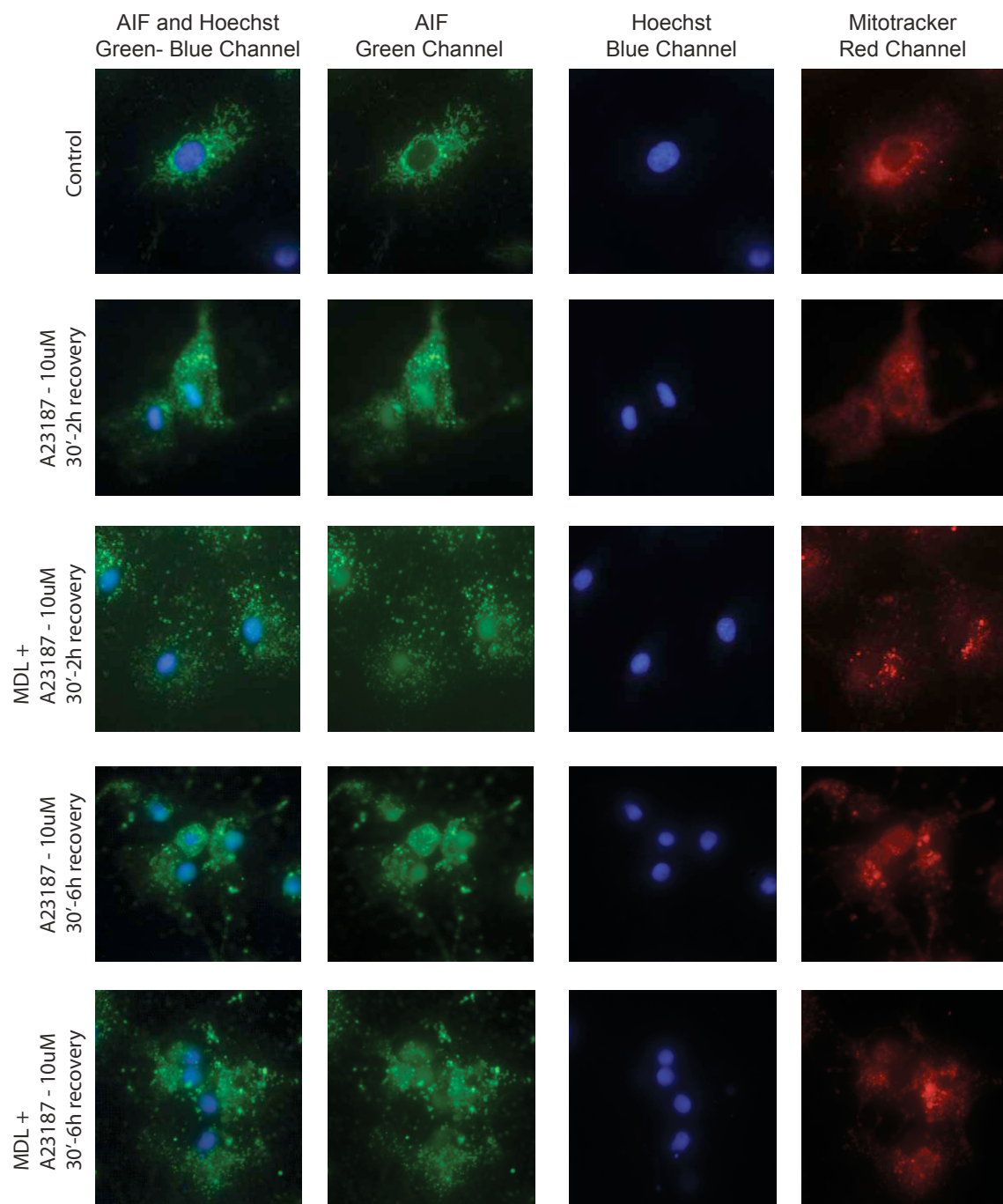


Fig 4.4 Thapsigargin-induced calpain activation is concomitant with AIF processing. SH-SY5Y cells were exposed to ER stressor Thapsigargin (100 μ M) for indicated time points. Protein extracts were analyzed by SDS-PAGE and western blotting done for (A) Calpain-1 and AIF, (B) Spectrin, and (D) AIF. No calpain activation or AIF proteolysis was observed at low dose thapsigargin. Increased proteolysis of spectrin, demonstrating robust calpain activation, and concomitant AIF processing was observed following high dose (100 μ M) of thapsigargin exposure at all time points. (C.) Representative images of tBOC assay. tBOC (a substrate used for assaying calpain activation) was used to analyze calpain activation. Images show robust calpain activation following high dose (100 μ M) thapsigargin exposure. Representative western blots and photomicrographs are shown. The data are from at least three independent experiments, represented as mean \pm S.E.M, * indicates $P < 0.05$ as per student's unpaired t-test, relative to control. Antibodies used: Calpain-1 (Abcam cat. no. ab28257 ; 1:2000), AIF (E-1 clone ; Santa cruz biotechnology cat. no. sc-13116 ; 1:1000), Spectrin (Millipore cat. no. MAB1622 ; 1:1000). Abbreviations: Cnt-Control, AIF-Apoptosis-inducing factor, Th-Thapsigargin.

Fig 4.4 Thapsigargin-induced calpain activation is concomitant with AIF processing.

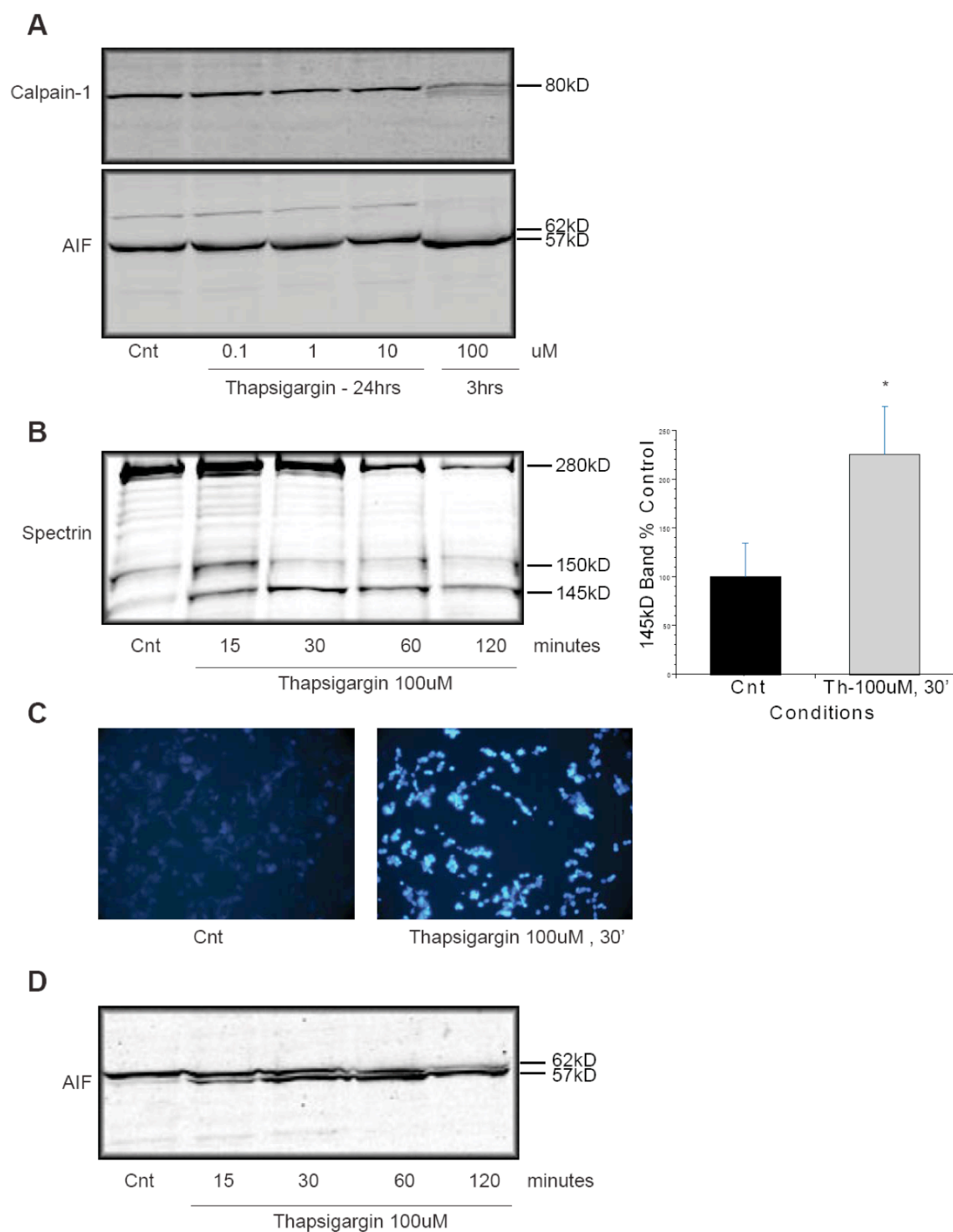
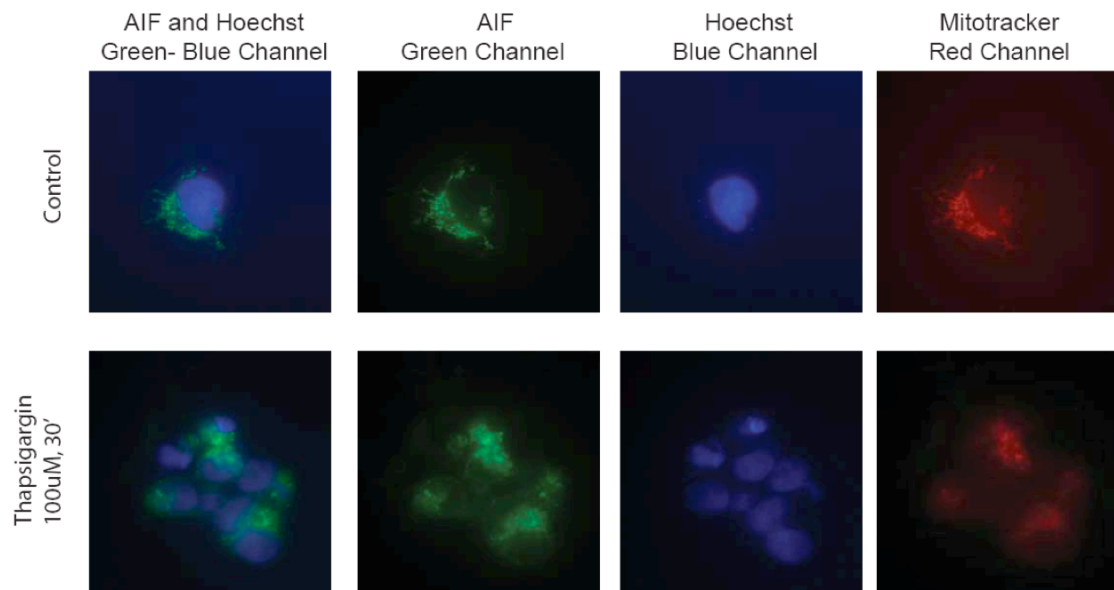


Fig 4.5 AIF shows cytosolic localization following high dose (100 μ M) Thapsigargin treatment. SH-SY5Y cells were exposed to the ER stressor Thapsigargin (100 μ M) for 30 min. Following incubation mitochondria were stained by mitotracker red 580 nm (1 mM) (Red channel) for 15 min, and then cells were fixed, permeabilized and immunostained for AIF (Green channel), and stained by Hoechst for nucleus (Blue channel). Representative confocal photomicrographs of neurons from three independent experiments are shown. Antibodies used: AIF (E-1 clone ; Santa cruz biotechnology cat. no. sc-13116 ; 1:50). Abbreviations: ER-Endoplasmic reticulum.

Fig 4.5 AIF shows cytosolic localization following high dose (100 μ M) Thapsigargin treatment.



Discussion

The results of this study show that calpain activation does not necessarily lead to AIF processing and nuclear translocation, but calpain may be one of the players for AIF processing. Further we show that AIF can translocate to the nucleus in mature 62 kD unprocessed form independent of calpain activity/peptidolytic processing.

Spectrin is the main component of the cell membrane cytoskeleton (Czogalla and Sikorski, 2005). It is a antiparallel heterodimer composed of 280 kD α and 247-460 kD β subunits, and exist as a tetramer in cytoskeleton (Czogalla and Sikorski, 2005). Spectrin is a calpain substrate (Siman et al., 1984; Nixon, 1986) and is routinely used as a marker for calpain activation (Wang, 2000). Excitotoxicity induces cell death by excessive release of glutamate (Sattler and Tymianski, 2001) and majority of this occurs by NMDA receptors (Wang et al., 2004). Primary hippocampal neuronal cultures demonstrated calpain activation, by appearance of 150 kD and 145 kD bands of spectrin breakdown, following NMDA exposure. AIF processing was not observed under these conditions. This suggests that calpain activation does not necessarily lead to AIF processing. Recently similar findings have been reported by Wang et al (Wang et al., 2009b) where NMDA treatment lead to calpain activation but AIF was not processed.

NMDA exposure did not induce AIF translocation to the nucleus or cytosolic distribution, instead AIF showed perinuclear notch localization along with mitochondria. Mitochondria move towards nucleus under stress (Landshamer et al., 2008) and necrotic signals (Laporte et al., 2007). Non translocation of AIF to the nucleus after NMDA treatment contrasts with findings of Wang et al. 2004, but that may be due to the difference in NMDA concentrations, length of treatment, and model system used. They use 500 μ M for 5 min, whereas we used 100 μ M for longer periods of time. Also, they used mice cortical cultures whereas our data is from rat hippocampal cultures. Overall, the lack of AIF translocation to the nucleus following NMDA exposure is in agreement with calpain's inability to process AIF.

Hippocampal neurons treated with A23187, like NMDA treatment, showed calpain activation, by appearance of 150 kD and 145 kD spectrin bands, and didn't demonstrate AIF processing. This suggests, like NMDA treatment, that calpain activation does not necessarily lead to AIF processing. But in contrast to NMDA, A23187 treatment demonstrated robust nuclear translocation of AIF. This suggests that AIF can translocate

to the nucleus in full-length 62 kD form without getting processed to 57 kD tAIF. This finding argues against the hypothesis that AIF has to be processed by a protease before it gets released from the mitochondria (Otera et al., 2005). Recently, Wang et al reported a similar finding regarding translocation of unprocessed 62 kD mature AIF to the nucleus in an middle cerebral artery (MCA) occlusion model of ischemia and NMDA treated primary neuronal cultures (Wang et al., 2009b). Possible mechanisms for mature 62 kD AIF translocation to the nucleus could be compromised interaction of AIF's hydrophobic domain with the mitochondrial inner membrane, severe breaches in the integrity of the mitochondrial inner membrane itself, changes in the redox state of the cell affecting stability of AIF, presence of free floating mature 62 kD AIF in the mitochondrial IMS or the presence of a second pool of AIF in the mitochondria in a different location (Wang et al., 2009b). We also observed that A23187 induced AIF nuclear translocation occurred in the presence of calpain inhibitor MDL28170, suggesting that AIF translocation can occur independent of calpain activity. It is in agreement with a recent observation that AIF translocates to the nucleus in calpain 4 knockout mice, which are functional calpain knockouts, following N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) or NMDA treatment (Wang et al., 2009b).

Besides mitochondria, endoplasmic reticulum (ER) is a major calcium buffering organelle (Verkhratsky, 2005). Mitochondrial networks are closely intertwined with ER tubules (Brookes et al., 2004; Pizzo and Pozzan, 2007) and VDAC physically interacts with IP3 via grp75 (Szabadkai et al., 2006). There is apoptotic cross talk (Hacki et al., 2000) and calcium cycling between mitochondria and ER (Mannella et al., 1998). The intracellular region between ER and mitochondria is a calcium hotspot (Pizzo and Pozzan, 2007)(Fig 1.3). Thus depletion of ER calcium store would locally increase calcium concentration in the mitochondria-ER region, with a possibility of activating mitochondrial μ -calpain. ER stressor thapsigargin, induces Ca^{2+} dysregulation by inhibiting the sarcoplasmic-endoplasmic reticulum Ca^{2+} -ATPase pump (SERCA) (Thastrup et al., 1990) in the SH-SY5Y cells (Nath et al., 1997) and induces cell death in SH-SY5Y cells by involvement of ER and mitochondria (Kitamura et al., 2003).

In SH-SY5Y cells we did not observe μ -calpain autoproteolysis at a low concentration (0.1 to 10 μM) of thapsigargin for up to 24 h but robust calpain activation was observed at higher concentration (100 μM) of thapsigargin demonstrated by μ -calpain autoproteolysis, spectrin breakdown products and tBOC assay. This indicates

that μ -calpain in the vicinity of mitochondria and ER is not easy to activate, suggesting the possibility of a local inhibitory regulation. Our data contrasts with that of Harriman et al (Harriman et al., 2002) where, in rabbit renal proximal tubules, low concentration (5 μ M) of thapsigargin induced calcium deregulation and hydrolysis of a calpain substrate - SLLVY-AMC within 2 min, demonstrating calpain activation. This could be explained by the fact that calpain substrate assays are not specific for μ -calpain.

In SH-SY5Y cells we did not observe AIF processing at low concentration (0.1 to 10 μ M) of thapsigargin for up to 24 h. In NIH3T3 cells AIF, along with caspase-12, is processed following treatment with low concentration (2 μ M) of thapsigargin for 48 h in a calpain dependent fashion with no involvement of executioner caspases (Sanges and Marigo, 2006). There was extensive cell death observed in SH-SY5Y cells following treatment for 48 h. It is also noteworthy that SH-SY5Y cells treated at lower dose (nM to 5 μ M) of thapsigargin for extended period of times have demonstrated caspase 2, 3, and 7 activation and protection by pretreatment with Z-VAD-FMK but not by calpain inhibitor III (Arai et al., 2004; Dahmer, 2005; Muruganandan and Cribb, 2006). This suggests that in neuroblastoma cells low dose thapsigargin induces a caspase-dependent apoptotic cascade. We observed AIF processing only at a very high concentration (100 μ M) of thapsigargin treatment, concomitant with robust and specific calpain activation demonstrated by autoproteolysis and 145 kD spectrin breakdown band. Pretreatment with MDL28170 (20 μ M) had no effect on AIF processing at high concentration (100 μ M) of thapsigargin treatment. One concern with the high concentration of thapsigargin is that thapsigargin could become non-specific and activate other cell death pathways besides calcium dysregulation such as unfolding protein response and/or ER-directed protein degradation by proteasome.

AIF translocates to the nucleus, along with caspase-12, following low dose (2 μ M) of thapsigargin treatment for 48 h in NIH3T3 cells in a calpain dependent fashion (Sanges and Marigo, 2006) but we were unable to demonstrate AIF nuclear translocation with low dose thapsigargin in the SH-SY5Y cells (results not shown). At the high concentration (100 μ M) of thapsigargin we did not detect AIF nuclear translocation rather detected only cytosolic distribution of AIF. Pretreatment with MDL28170 (20 μ M) had no effect on the AIF redistribution following high concentration (100 μ M) of thapsigargin treatment. As the length and strength of an insult determines which cell

death cascades are activated (Meli et al., 2004), AIF processing and distribution may also depend upon the cell type and insult strength and duration.

BCL-2 family of proteins regulates AIF translocation (Cregan et al., 2002). Apoptosis-inducing factor (AIF) proteolytic processing and mitochondrial release were both inhibited by overexpression of BCL-2 and BCL-XL (Daugas et al., 2000b; Otera et al., 2005). Bid inhibitor and Bid siRNA prevented the AIF release and translocation in HT-22 neurons (Landshamer et al., 2008). These literature evidence suggests that outer mitochondrial membrane permeabilization is upstream of AIF processing and nuclear translocation, indicating necessity for import of something from cytosol for AIF processing and subsequent nuclear translocation. Possibility of calpain (cytosolic μ -calpain) getting translocated to the mitochondria and playing a role in AIF processing and nuclear translocation has been addressed in this chapter to a certain extent. Our results demonstrate that calpain activation does not necessarily lead to AIF processing, AIF can translocate to the nucleus independent of calpain activation and as a full length mature 62 kD AIF without any processing. These results also show that calpain activation and AIF processing are concomitant under stringent ER stress signals. This suggests a possibility of calpain mediated AIF processing but whether calpain is directly involved in AIF processing or is indirectly processing AIF via lysosomal cathepsins needs further investigation. Since mitochondrial outer membrane permeabilization is upstream of AIF processing and nuclear translocation, other possibilities such as mitochondrial translocation of an activator of endogenous AIF cleaving mitochondrial protease, mitochondrial release of the AIF cleaving protease's inhibitor (Modjtahedi et al., 2006) or import of additional pro-apoptotic cytosolic factors to the mitochondria that could facilitate AIF processing and nuclear translocation also needs further investigation. Finally, there could be multiple mechanisms for the processing and distribution (nuclear or cytosolic) of AIF in the cells, certainly at least one without involvement of calpains.

Chapter Five

Calpastatin localization in the mitochondrial intermembrane space

Aashish Joshi, Vimala Bondada, Dingyuan Lou, and James W. Geddes

"The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' but 'That's funny...'- Isaac Asimov

Introduction

The calpain proteolytic system is comprised of the calpain family of cysteine proteases, their specific endogenous inhibitor calpastatin (Dayton et al., 1976; Croall and DeMartino, 1991; Mellgren, 1991; Brustis et al., 1994; De Jongh et al., 1994; Shea et al., 1995) and activator calcium (Goll et al., 2003). Calpastatin inhibits calpains in a reversible calcium dependent manner (Cottin et al., 1981; Imajoh and Suzuki, 1985; Otsuka and Goll, 1987; Wendt et al., 2004; Hanna et al., 2007; Hanna et al., 2008). Calpastatin, being an indispensable component of the calpain proteolytic system, plays a key regulatory role in various physiological functions such as cell cycle progression (Zhang et al., 1997), proliferation (Zhang et al., 1996), differentiation (Grynspan et al., 1997a; Patel and Lane, 2000), migration (Franco et al., 2004; Satish et al., 2005; Wu et al., 2006), synaptic remodeling and long term potentiation (Tomimatsu et al., 2002), embryonic development (Arthur et al., 2000), meiosis and mitosis (Schollmeyer, 1988; Santella et al., 2000) as well as pathological states including muscular dystrophy (Richard et al., 1995), diabetes (Horikawa et al., 2000), Alzheimer's disease (Grynspan et al., 1997b; Tsuji et al., 1998), cataract (Nakamura et al., 2000), multiple sclerosis (Shields et al., 1999), neuronal ischemia (Saido et al., 1993b; Roberts-Lewis et al., 1994), obsessive compulsive disorder (Mundo et al., 1997) and traumatic brain and spinal cord injuries (Springer et al., 1997; Pike et al., 2001; Goll et al., 2003; Liu et al., 2004).

The single calpastatin molecule has four inhibitory repeats, capable of inhibiting one calpain molecule each (Maki et al., 1987; Goll et al., 2003) and an N-terminal L domain which lacks inhibitory activity (Averna et al., 2001). An additional N-terminal XL domain has been identified in bovine calpastatin (Cong et al., 1998). There are various isoforms of calpastatin with different domain combinations due to alternative splicing (Lee et al., 1992a; Lee et al., 1992b; Takano et al., 2000), exon skipping (De Tullio et al., 2007), and usage of different promoters (Imajoh et al., 1987; Cong et al., 1998). Multiple

isoforms are found in a single tissue (Melloni et al., 1998b). Molecular weight range of the various calpastatin isoforms is 17.5 kD to 85 kD (Goll et al., 2003) and they migrate aberrantly on SDS-PAGE (Takano and Murachi, 1982a; Maki et al., 1991). The functional significance of so many different isoforms is not known. Calpastatin's inhibitory activity is regulated by protein's abundance and by post translational modifications (Cong et al., 1998) including phosphorylation (Salamino et al., 1994; Salamino et al., 1997). There might be additional components to the regulation of calpastatin activity, one possible mechanism being the differential subcellular compartmentalization of different isoforms of calpastatin.

Calpastatin was thought to be primarily a cytosolic protein (Taylor et al., 1991; Kumamoto et al., 1995). In SH-SY5Y cells 90% of calpastatin is cytosolic (Grynspan et al., 1997a). Recently along with calpain, calpastatin was found to be associated with ER and golgi fractions (Hood et al., 2003; Hood et al., 2004). It is reported that calpastatin is associated with the inner mitochondrial membrane of the pulmonary smooth muscle mitochondria (Kar et al., 2007; Kar et al., 2008) however other studies did not find calpastatin in the mitochondria (Ozaki et al., 2007). We recently localized μ -calpain to the mitochondrial intermembrane space (Badugu et al., 2008) and hypothesized that this location would protect μ -calpain from calpastatin, thus making its activation easier. We observed that mitochondrial μ -calpain requires high calcium levels (in mM) for activation (Joshi et al., 2009). The reason behind this high calcium requirement was not determined, with one possibility being the presence of a calpain inhibitor, calpastatin, in the neuronal mitochondria.

The goal of this study was to evaluate the hypothesis that calpastatin is present in the neuronal mitochondria. In the present study we have identified calpastatin in the mitochondrial intermembrane space of the mitochondria isolated from the rat cerebral cortex and SH-SY5Y cells. Further our results demonstrate that the N-terminal first 30 amino acids of calpastatin's XL domain are a mitochondrial targeting sequence. Identification of calpastatin in the mitochondrial intermembrane space and unraveling its possible mechanism of import into mitochondria provides a novel mechanism for regulating the activity of mitochondrial μ -calpain in the mitochondrial intermembrane space.

Results

Calpastatin is associated with the mitochondria

Purified μ -calpain is activated at micromolar calcium concentrations (Suzuki, 1991). We observed that mitochondrial μ -calpain requires high calcium levels (5 mM) for activation (Fig 5.1) (Joshi et al., 2009). Thus, we probed for the calpain inhibitor - calpastatin, in mitochondria-enriched fraction of the SH-SY5Y cells and rat cerebral cortex. Calpastatin immunoreactivity was stronger in the mitochondria-enriched fraction from both SH-SY5Y cells (Fig 5.2) and rat cerebral cortex (Fig 5.6), relative to the corresponding homogenate samples.

Calpastatin is located in the mitochondrial intermembrane space

To determine whether calpastatin is inside the mitochondria or is associated on the outer face of the outer mitochondrial membrane, mitochondria-enriched fractions of the SH-SY5Y cells and rat cerebral cortex were incubated with enzyme proteinase K. The calpastatin results were compared to AIF, which is located inside the mitochondrial outer membrane. Calpastatin and AIF immunoreactivity persisted following proteinase K incubation in the SH-SY5Y mitochondria (Fig 5.3). Calpastatin immunoreactivity persisted following proteinase K incubation in the rat cerebral cortex mitochondria (Fig 5.7). This suggests that calpastatin is localized inside the outer mitochondrial membrane. To confirm the activity of proteinase K, mitochondrial fractions were solubilized with 0.2% Triton-X 100 and then incubated with proteinase K. Both calpastatin and AIF immunoreactivity were lost (Fig 5.3 and Fig 5.7) demonstrating that proteinase K was active.

To determine the submitochondrial localization of calpastatin, Percoll-enriched mitochondria of the SH-SY5Y cells and rat cerebral cortex were subfractionated via hypotonic rupture into their components namely Mitoplasts (inner mitochondrial membrane and matrix), Intermembrane space, and Outer mitochondrial membrane. Calpastatin immunoreactivity was found in the mitoplast and intermembrane space fractions but not in the outer mitochondrial membrane fraction in both SH-SY5Y cells (Fig 5.4) and rat cerebral cortex (Fig 5.8). AIF immunoreactivity, consistent with its localization, was observed in the mitoplast fraction but not in the outer mitochondrial membrane fraction or intermembrane space fraction (Fig 5.4 and Fig 5.8).

To determine whether calpastatin is inside the mitoplast (i.e. matrix) or is associated with the outer face of the inner mitochondrial membrane, SH-SY5Y cells mitoplasts were incubated with proteinase K. Calpastatin immunoreactivity was lost following proteinase K incubation, suggesting that calpastatin is associated with the outer face of the inner mitochondrial membrane (Fig 5.5 A). AIF was also digested in mitoplasts with proteinase K, observed as faster migrating band (Fig 5.5 B), though the digestion was not complete as AIF is a trans inner membrane protein. Matrix protein mHSP70, as expected, was protected from proteinase K digestion (Fig 5.5 C). To confirm the activity of proteinase K, mitoplast fractions were solubilized with 0.2% Triton-X 100 and then incubated with proteinase K. Calpastatin, AIF and mHSP70 immunoreactivities were lost following solubilization indicating that proteinase K was active (Fig 5.5 A, B and C).

Mitochondrial targeting sequence in calpastatin

The majority of mitochondrial proteins, including all IMS proteins, are nuclear encoded, synthesized in the cytosol, and subsequently imported from the cytosol into the mitochondria (Anderson et al., 1981; Poyton and McEwen, 1996; Scarpulla, 2002). There are various mechanisms of mitochondrial protein import. Large IMS proteins are imported by interaction with TOM and TIM, via an N-terminal mitochondrial targeting sequence (Herrmann and Hell, 2005). *In silico* sequence analysis of the calpastatin protein using four different programs– iPSORT (Bannai et al., 2002), SLPFA (Tamura and Akutsu, 2007), Target P 1.1 server (Emanuelsson et al., 2007), and MitoProt II-v1.101 (Claros and Vincens, 1996) predicted mitochondrial localization of calpastatin (Fig 5.9 A), with first 30 amino acids of the XL-domain of calpastatin being the mitochondrial targeting sequence (Fig 5.9 B). There was 73.3% sequence identity between first 30 amino acids of rat and human calpastatin sequence, suggesting possibly the same mechanism of calpastatin import to mitochondria in these two species (Fig 5.9 C). Based on the subcellular location prediction programs we incorporated first 30 amino acids of the XL domain of rat calpastatin (1-30XLDCAST) into a mammalian GFP expression vector - pWPXL. 1-30XLDCAST-pWPXL vector when transfected to SH-SY5Y cells had a tubular distribution which co-localized with mitochondrial dye mitotracker red (Fig 5.10), suggesting that the first 30 amino acids of the XL domain of calpastatin are a mitochondrial targeting sequence.

Fig 5.1 Mitochondrial μ -calpain requires high calcium concentration for activation. Percoll-enriched SH-SY5Y cells mitochondria were incubated with two different concentrations of calcium in Tris-HCl based calpain activity buffer for 2 h at room temperature. Samples were probed for μ -calpain activation by western blotting using either calpain-1 domain III antibody or N-terminal calpain-1 antibody. Calpain activation results in autoproteolysis of calpain at N-terminus. Thus, calpain activation is visualized either as a faster migrating band with domain III antibody or as a loss of immunoreactivity with N-terminal antibody. Mitochondrial μ -calpain was not activated with 0.5 mM Ca^{2+} incubation, but was activated following 5 mM Ca^{2+} incubation. Representative western blots are shown. Antibodies used: μ -calpain domain III (Calbiochem cat. no. 208728 ; 1:2000), μ -calpain N-terminal (Abcam cat. no. ab28257 ; 1:2000). Abbreviations: Cnt-Control.

Fig 5.1 Mitochondrial μ -calpain requires high calcium concentration for activation.

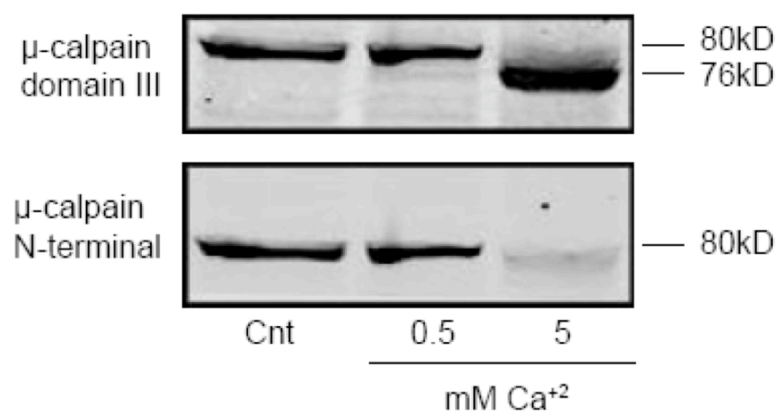


Fig 5.2 Calpastatin is associated with the mitochondrial fraction from the SH-SY5Y cells. The starting homogenate and the Percoll-enriched mitochondria from SH-SY5Y cells were analyzed for the presence of calpastatin by SDS-PAGE and western blotting. Mitochondrial fraction showed enrichment of calpastatin immunoreactivity relative to homogenate. Equal amounts of protein were loaded in each lane as determined by BCA protein assay. Representative western blot is shown. Antibodies used: Calpastatin (Chemicon cat. no. MAB3084 ; 1:1000). Abbreviations: CAST-Calpastatin, H-Homogenate, M-Mitochondrial fraction.

Fig 5.2 Calpastatin is associated with the mitochondrial fraction from the SH-SY5Y cells.

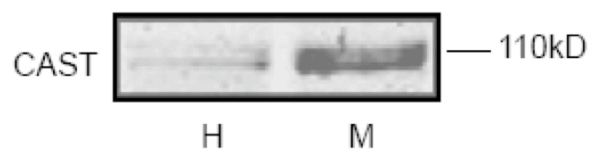


Fig 5.3 Calpastatin is located inside the mitochondria from the SH-SY5Y cells. Percoll-enriched mitochondria from the SH-SY5Y cells were incubated with proteinase K (50 µg/ml) for 30 min at 37 °C, with or without 0.2% Triton-X 100 solubilization. PMSF (2 mM) was added to the reaction to terminate proteinase K activity. Samples were resolved by SDS-PAGE and analyzed by western blotting for calpastatin and AIF. (A.) Calpastatin immunoreactivity persisted in non-solubilized proteinase K treated mitochondria, but was lost in 0.2% Triton-X 100 solubilized proteinase K treated mitochondria. (B.) AIF, an intramitochondrial protein, was protected from proteinase K digestion in non-solubilized mitochondria. This suggests calpastatin is present inside the outer mitochondrial membrane. Representative western blots are shown. Antibodies used: Calpastatin (Chemicon cat. no. MAB3084 ; 1:1000), AIF (E-1 clone ; Santa cruz biotechnology cat. no. sc-13116 ; 1:1000). Abbreviations: CAST-Calpastatin, AIF-Apoptosis-inducing factor, PK-Proteinase K, Tx-0.2% Triton-X 100, Cnt-Control.

Fig 5.3 Calpastatin is located inside the mitochondria from the SH-SY5Y cells.

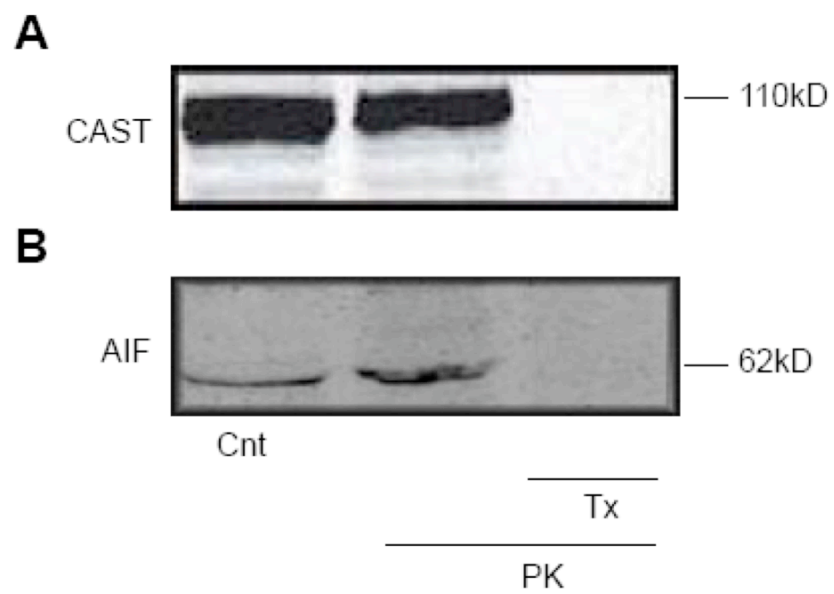


Fig 5.4 Calpastatin is present in the mitochondrial intermembrane space and mitoplast fractions from the SH-SY5Y cells. The outer mitochondrial membrane of Percoll-enriched mitochondria from SH-SY5Y cells was disrupted by incubation in a hypotonic (10 mM HEPES/KOH, pH 7.4) solution at 4 °C for 20 min. Mitoplasts (MP) were pelleted by centrifugation at 1900 g for 15 min at 4 °C. The supernatant fraction was further centrifuged at 35,000 g for 15 min at 4 °C to pellet the outer mitochondrial membrane fragments (OMM) and supernatant fraction was the intermembrane space (IMS). Samples were resolved by SDS-PAGE and analyzed by western blotting for calpastatin and AIF. (A.) Calpastatin immunoreactivity was detected in the mitochondria, mitoplast and intermembrane space fraction but not in the mitochondrial outer membrane fraction. (B.) AIF immunoreactivity was detected in the mitochondria and mitoplast fraction. Representative western blots are shown. Antibodies used: Calpastatin (Chemicon cat. no. MAB3084 ; 1:1000), AIF (E-1 clone ; Santa cruz biotechnology cat. no. sc-13116 ; 1:1000). Abbreviations: CAST-Calpastatin, AIF-Apoptosis-inducing factor, M-Mitochondrial fraction, MP-Mitoplast, IMS-Intermembrane Space, OMM-Outer mitochondrial membrane fraction.

Fig 5.4 Calpastatin is present in the mitochondrial intermembrane space and mitoplast fractions from the SH-SY5Y cells.

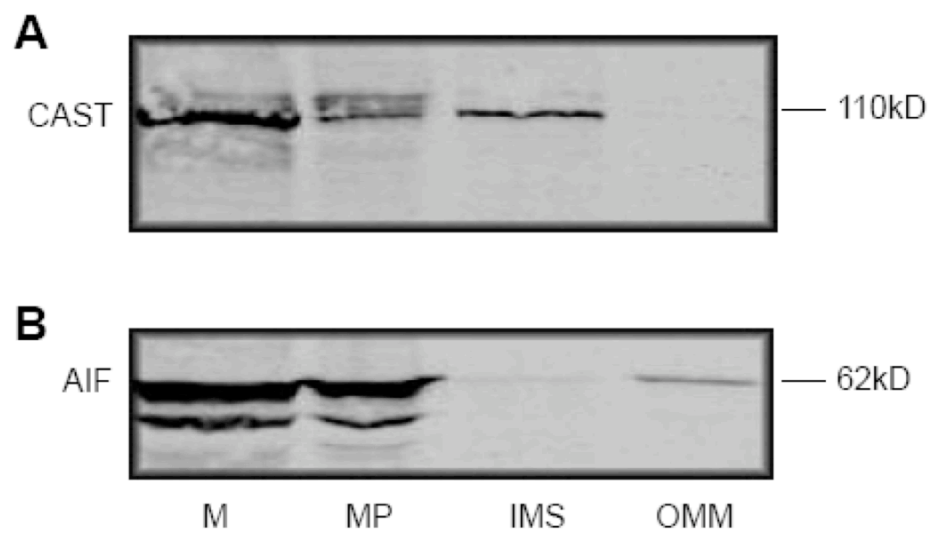


Fig 5.5 Calpastatin is located on the inner mitochondrial membrane in the SH-SY5Y cells mitochondria. The outer mitochondrial membrane of Percoll-enriched mitochondria from SH-SY5Y cells was disrupted by incubation in a hypotonic (10 mM HEPES/KOH, pH 7.4) solution at 4 °C for 20 min. Mitoplasts (MP) were pelleted by centrifugation at 1900 g for 15 min at 4 °C. Mitoplasts were then incubated with proteinase K (50 µg/ml) for 30 min at 37 °C, with or without 0.2% Triton-X 100 solubilization. PMSF (2 mM) was added to the reaction to terminate proteinase K activity. Samples were resolved by SDS-PAGE and analyzed by western blotting for calpastatin, mHSP70 and AIF. (A.) Calpastatin immunoreactivity was lost following proteinase K incubation in both non-solubilized mitoplasts and 0.2% Triton-X 100 solubilized mitoplasts. (B.) mHSP70, a matrix protein, was protected from proteinase K digestion in non-solubilized mitoplasts. (C.) AIF, an inner membrane protein, was processed with proteinase K digestion in non-solubilized mitoplasts. This suggests calpastatin is primarily present in the mitochondrial intermembrane space and on the outer face of the inner mitochondrial membrane. Representative western blots are shown. Antibodies used: Calpastatin (Chemicon cat. no. MAB3084 ; 1:1000), mHSP70 (Affinity Bioreagents cat. no. MA3-028 ; 1:10000), AIF (E-1 clone ; Santa cruz biotechnology cat. no. sc-13116 ; 1:1000). Abbreviations: CAST-Calpastatin, AIF-Apoptosis-inducing factor, mHSP70-Mitochondrial heat shock protein 70, PK-Proteinase K, Tx-Triton-X 100 and Cnt-Control.

Fig 5.5 Calpastatin is located on the inner mitochondrial membrane in the SH-SY5Y cells mitochondria.

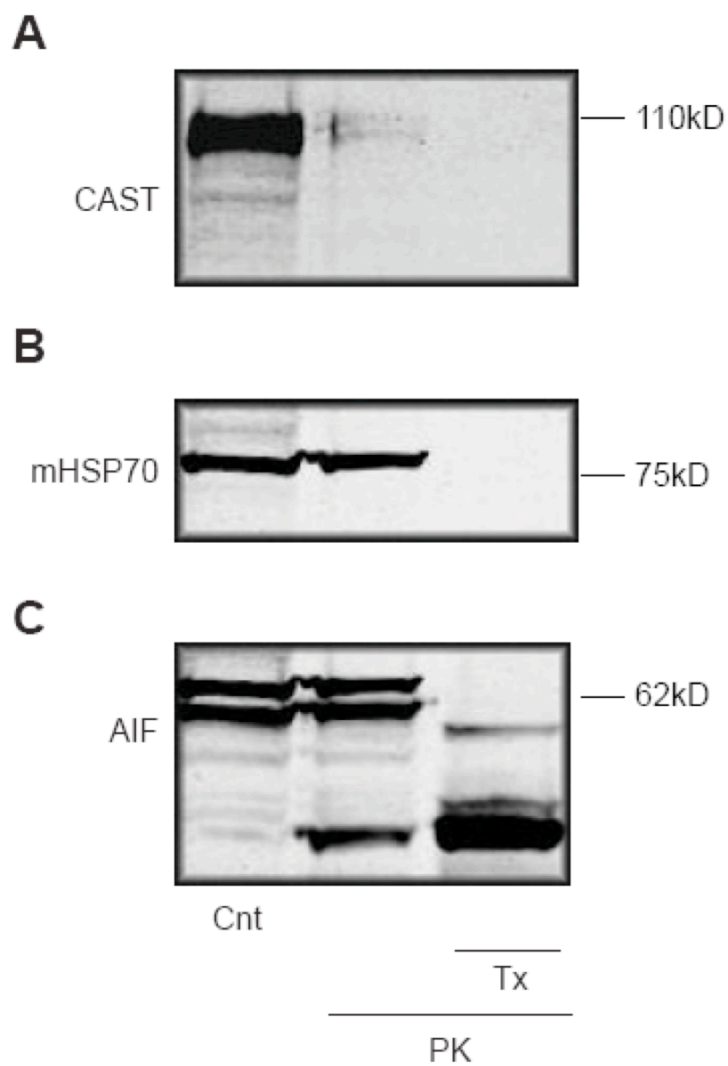


Fig 5.6 Calpastatin is associated with the mitochondrial fraction from the rat cerebral cortex. The starting homogenate, after differential fraction and the Percoll-enriched mitochondrial fraction from rat cerebral cortex were analyzed for the presence of calpastatin by SDS-PAGE and western blotting. Mitochondrial fraction showed enrichment of calpastatin immunoreactivity relative to homogenate and after differential fractions. Equal amount of protein was loaded in each lane as determined by BCA protein assay. Representative western blot is shown. Antibodies used: Calpastatin (Chemicon cat. no. MAB3084 ; 1:1000). Abbreviations: CAST-Calpastatin, H-Homogenate, D-After differential fraction, M-Mitochondrial fraction.

Fig 5.6 Calpastatin is associated with the mitochondrial fraction from the rat cerebral cortex.

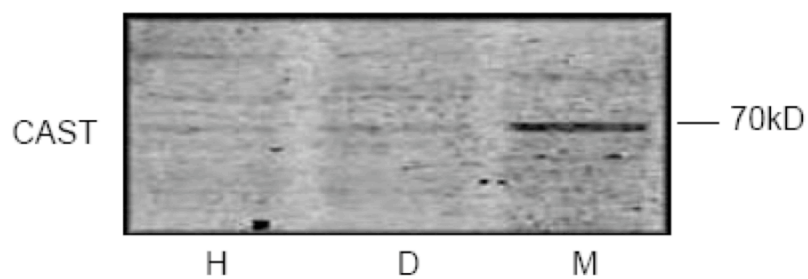


Fig 5.7 Calpastatin is located inside the mitochondria from the rat cerebral cortex. Percoll-enriched mitochondria from rat cerebral cortex were incubated with proteinase K (50 µg/ml) for 30 min at 37 °C, with or without 0.2% Triton-X 100 solubilization. PMSF (2 mM) was added to the reaction to terminate proteinase K activity. Samples were resolved by SDS-PAGE and analyzed by western blotting for calpastatin. Calpastatin immunoreactivity persisted in non-solubilized proteinase K treated mitochondria, but was lost in 0.2% Triton-X 100 solubilized proteinase K treated mitochondria. This suggests calpastatin is present inside the outer mitochondrial membrane. Representative western blot is shown. Antibodies used: Calpastatin (Chemicon cat. no. MAB3084 ; 1:1000). Abbreviations: CAST-Calpastatin, PK-Proteinase K, Tx-0.2% Triton-X 100, Cnt-Control.

Fig 5.7 Calpastatin is located inside the mitochondria from the rat cerebral cortex.

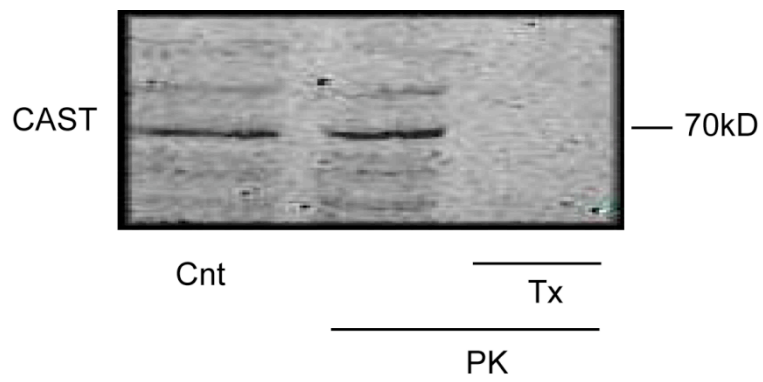


Fig 5.8 Calpastatin is present in the mitochondrial intermembrane space and mitoplast fractions from the rat cerebral cortex. The outer mitochondrial membrane of Percoll-enriched mitochondria from rat cerebral cortex was disrupted by incubation in a hypotonic (10 mM HEPES/KOH, pH 7.4) solution at 4 °C for 20 min. Mitoplasts (MP) were pelleted by centrifugation at 1900 g for 15 min at 4 °C. The supernatant fraction was further centrifuged at 35,000 g for 15 min at 4 °C to pellet the outer mitochondrial membrane fragments (OMM) and supernatant fraction was the intermembrane space (IMS). Samples were resolved by SDS-PAGE and analyzed by western blotting for calpastatin and AIF. (A.) Calpastatin immunoreactivity was detected in the mitochondria, mitoplast and intermembrane space fraction but not in the mitochondrial outer membrane fraction. (B.) AIF immunoreactivity was detected in the mitochondria and mitoplast fraction. Representative western blots are shown. Antibodies used: Calpastatin (Chemicon cat. no. MAB3084 ; 1:1000), AIF (E-1 clone ; Santa cruz biotechnology cat. no. sc-13116 ; 1:1000). Abbreviations: CAST-Calpastatin, AIF-Apoptosis-inducing factor, M-Mitochondrial fraction, MP-Mitoplast, IMS-Intermembrane Space, OMM-Outer mitochondrial membrane fraction.

Fig 5.8 Calpastatin is present in the mitochondrial intermembrane space and mitoplast fractions from the rat cerebral cortex.

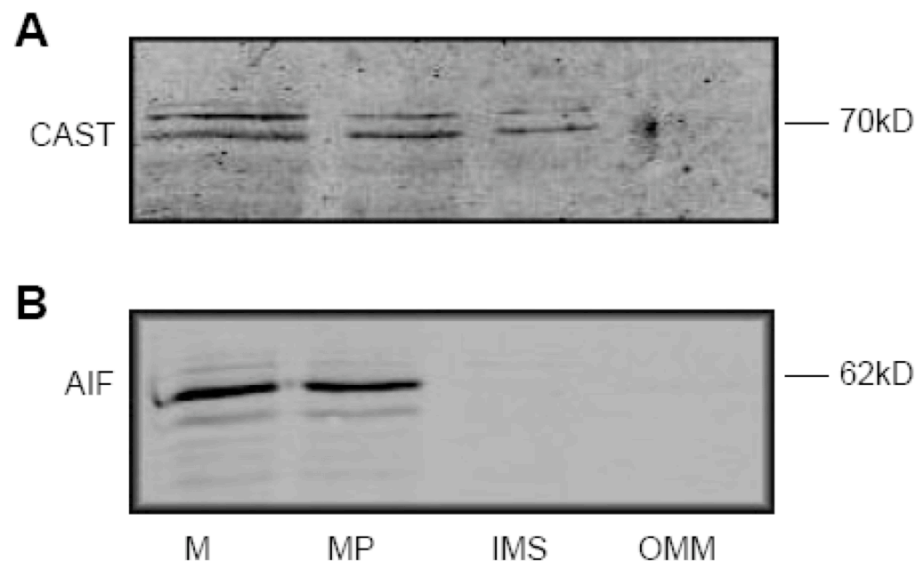


Fig 5.9 N-terminal 1-30 amino acids of the XL domain of calpastatin are predicted to be a mitochondrial targeting sequence. (A.) *In silico* sequence analysis of calpastatin by four different softwares. All of them predict mitochondrial localization of calpastatin. (B.) First 30 amino acids of the XL domain of calpastatin are predicted as a mitochondrial targeting sequence. (C.) There is 73.3% identity in the first 30 amino acids of rat and human calpastatin sequence.

Fig 5.9 N-terminal 1-30 amino acids of the XL domain of calpastatin are predicted to be a mitochondrial targeting sequence.

A

Sequence Analysis - Rat calpastatin isoform A

A.) SLPFA	Mitochondrial (mTP) SVM score=2.772510
B.) Target P 1.1 server	Mitochondrion mTP score=0.727
C.) iPSORT	It has mitochondrial targeting sequence
D.) MitoProt II – v1.101	Probability of export to mitochondria =0.4926

B

Mitochondrial targeting sequence – XL domain

1 **MSRPG PKPAA SSRPR RGAAA SHTQE HVNEK**
NIGSS SKPAEEEVPK PKVDE DAT 701

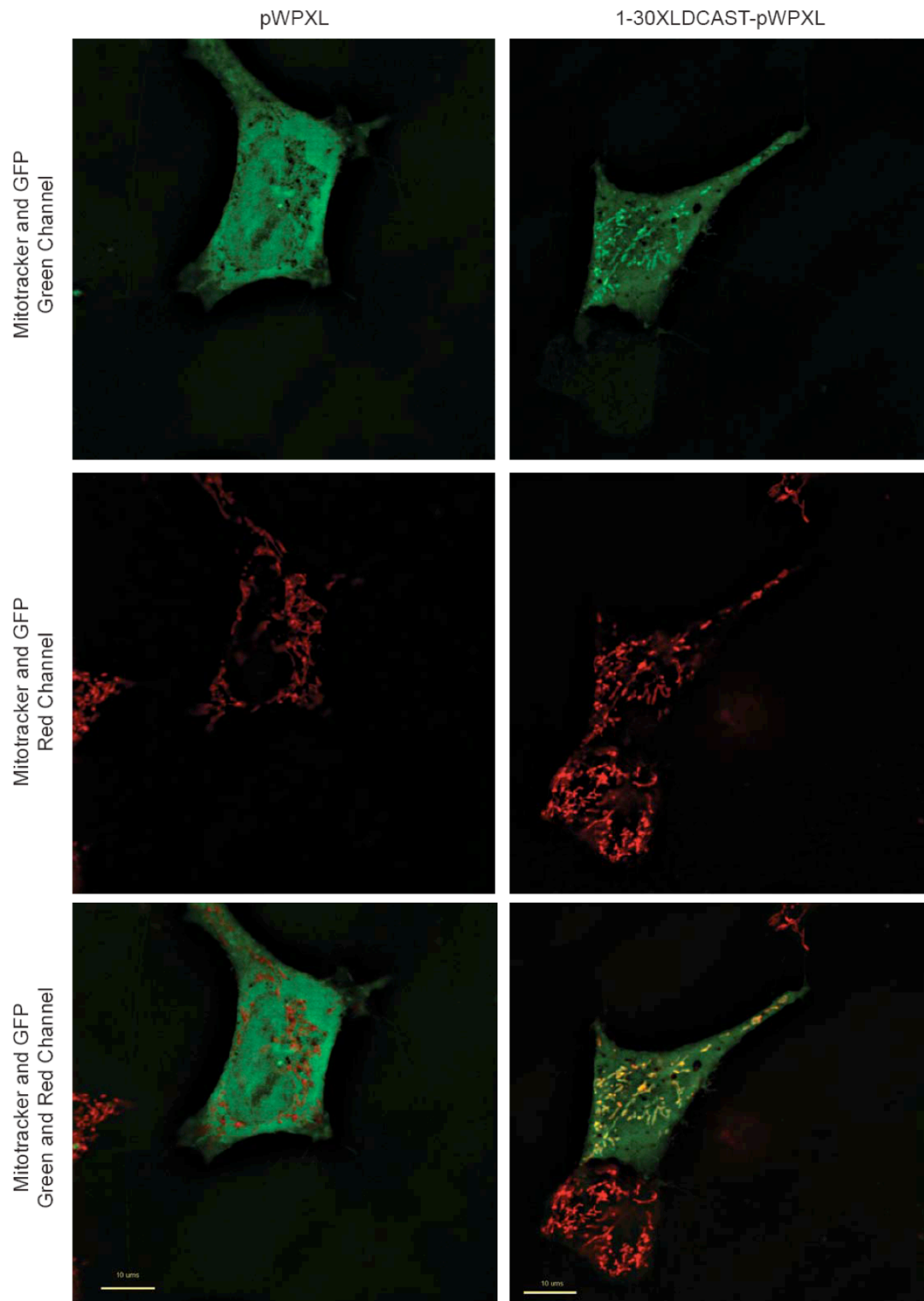
C

Rat MSRPG**PK**PAASSRPRRGAAA**SHTQE**HVNEK
Human MSQPG**Q**KPAASPRPRRAAA**ARRT**HEHV**SEK**

73.3% identity in 30 residues overlap (SIM alignment tool)

Fig 5.10 1-30XLDCAST-pWPXL shows mitochondrial localization. N-terminal first 30 amino acids of the XL domain of rat calpastatin were PCR amplified and ligated into a mammalian GFP expression vector. Transfection of this GFP tagged first 30 amino acids of the XL domain of calpastatin (1-30XLDCAST-pWPXL) into SH-SY5Y cells showed tubular localization that co-localized with the mitochondrial stain mitotracker red. Representative confocal images are shown. Abbreviations: PCR-Polymerase chain reaction, GFP-Green fluorescent protein, pWPXL-Plasmid WPXL, 1-30XLDCAST-First 30 amino acids XL domain calpastatin.

Fig 5.10 1-30XLDCAST-pWPXL shows mitochondrial localization.



Discussion

The purpose of this study was to examine the hypothesis that the calpain inhibitor, calpastatin, is present in the CNS mitochondria. Results of this study demonstrate that calpastatin is associated with the Percoll-enriched mitochondria from SH-SY5Y neuroblastoma cells and rat cerebral cortex. Further we show that calpastatin is located in the mitochondrial intermembrane space by mitochondrial subfractionation and proteinase K protection assay. The results also demonstrate that the first 30 amino acids of calpastatin's XL domain act as a mitochondrial targeting sequence.

Calpastatin immunoreactivity was significantly enriched in the Percoll-enriched mitochondrial fraction of SH-SY5Y cells and rat cerebral cortex, relative to corresponding homogenates, demonstrating the presence of calpastatin in the mitochondria. Previously calpastatin was predominantly localized to the cytosol (Takano and Murachi, 1982b; Adachi et al., 1991; Taylor et al., 1991; Kumamoto et al., 1997; Tullio et al., 1999; Ozaki et al., 2007). Recently calpastatin has been reported to be present in various subcellular compartments along with calpain (Barnoy et al., 1999; Hood et al., 2003; Hood et al., 2004; Haim et al., 2006). Calpastatin immunoreactivity was protected by proteinase K digestion of mitochondria implying its location to be internal to the mitochondrial outer membrane. We further explored the submitochondrial localization of calpastatin by submitochondrial fractionation using HEPES based hypo-osmotic shock. Calpastatin was found in the mitochondrial intermembrane space and mitoplasts but not in the outer mitochondrial membrane. Mitoplast associated calpastatin was digested by proteinase K suggesting it is localized on the outer face of the mitochondrial inner membrane. Calpastatin has been demonstrated to be located on the pulmonary smooth muscle mitochondrial inner membrane facing intermembrane space (Kar et al., 2008). This is in agreement with our finding of calpastatin in the mitochondrial intermembrane space and inner mitochondrial membrane fractions from SH-SY5Y cells and rat cerebral cortex.

The presence of calpastatin in the mitochondrial intermembrane space, the same submitochondrial compartment as mitochondrial μ -calpain, helps to explain various observations. The lack of involvement of mitochondrial μ -calpain in AIF processing following mPTP (Joshi et al., 2009) could be due to the presence of calpastatin in the same mitochondrial compartment as μ -calpain. Release of AIF from the isolated brain mitochondria required addition of exogenous calpain (Polster et al., 2005), which could

also be due to the presence of calpastatin within mitochondria. This also explains why mitochondrial μ -calpain requires high calcium (mM) for activation.

Rat brain has multiple mRNA's for calpastatin (De Tullio et al., 1998) encoding different isoforms of calpastatin. Calpastatin is present in rat brain as at least six alternatively spliced isoforms having combinations of XL, L, and four inhibitory repeats (De Tullio et al., 1998). Such heterogeneity in a single protein suggests likelihood of functional significance of the different isoforms, which is as yet unknown. One possibility is that the different isoforms are modified differentially by post-translational modifications to cater to various cellular needs. An alternate possibility is that different domain combinations target calpastatin to different subcellular locations.

XL domain containing calpastatin, similar to type I calpastatin, has been recently identified in the rat brain (De Tullio et al., 2007). XL domain containing calpastatin has various combinations of exons 4, 6, and 8. XL domain of calpastatin does not affect calpain calpastatin complex formation in the absence of calcium (De Tullio et al., 2007). Thus the function of XL domain of CAST has yet not been identified. Calpastatin is not encoded in the mitochondria, like most mitochondrial proteins it has to be imported from the cytosol. The mechanism of calpastatin import to the mitochondria is also unresolved so far. A chimeric protein consisting of the N-terminus 30 amino acids of XL domain of calpastatin and GFP showed mitochondrial localization, suggesting that first 30 amino acids of XL domain of calpastatin are a mitochondrial targeting sequence. This ascribes function to the XL domain of calpastatin. Whether calpastatin is exclusively in the intermembrane space or it is an inner mitochondrial membrane anchored protein remains purely speculative at this point. Class I intermembrane space proteins have a bipartite sequence (Herrmann and Hell, 2005). They are imported via a leading N-terminus mitochondrial targeting sequence, which may be processed by a protease in the matrix, and second hydrophobic sorting domain which locks protein in the inner mitochondrial membrane (Herrmann and Hell, 2005). Though calpastatin has a leading N-terminus mitochondrial targeting sequence and a predicted cleavage site at the 18th amino acid position it lacks an amphipathic helix in the N-terminus and a hydrophobic sorting domain on the hydropathicity plot. Further we noticed that AIF, which is an inner mitochondrial membrane anchored protein, is not found in the IMS fraction on mitochondrial subfractionation of SH-SY5Y cellular or rat cerebral cortex mitochondria but calpastatin, unlike AIF, is detected in the IMS fraction. Calpastatin has a stretch of

positively charged amino acids lys-lys-arg-his-lys-lys in the L – domain. Thus it is highly likely that calpastatin electrostatically binds to the mitochondrial inner membrane (Hood et al., 2004) and is not a membrane anchored protein.

The efficiency and level of calpastatin are crucial in regulating calpain activity (Pontremoli et al., 1988), but the isoform variability may be an additional regulatory component by preferentially targeting XL domain containing calpastatin to the mitochondrial intermembrane space. It is highly likely that other isoforms thus may be targeted to other subcellular locations, which needs further investigation. Transcriptional modulation of calpastatin isoforms may thus modulate the amount of calpastatin in various subcellular compartments including mitochondria. Hence this could be a novel mechanism that ultimately effects localized calpain activation and the outcome thereafter, producing varied pathology and physiological response of a cell to similar levels of increased calcium.

The immunoreactivity of mitochondrial calpastatin in the SH-SY5Y cells was detected at 110 kD. Cong et al (Cong et al., 1998) showed that the XL domain containing calpastatin runs at 145 kD. One possible explanation could be a processing event after mitochondrial import. Mitochondrial proteins imported by mechanism of N-terminal mitochondrial targeting sequence are processed by a matrix protease after the import (Herrmann and Hell, 2005). Thus XL domain calpastatin might undergo similar cleavage following import and thus runs at a lower molecular weight. Rat cerebral cortex mitochondrial calpastatin was detected at 70 kD. Mitochondrial μ -calpain may get activated during or prior to the isolation procedure (Garcia et al., 2005). Calpastatin is a calpain substrate (DeMartino et al., 1988; Carragher et al., 2002; Goll et al., 2003) and calpastatin can be degraded due to ischemia by calpain into a 50 kD protein (Blomgren et al., 1999). It is possible that mitochondrial calpastatin gets proteolysed by mitochondrial μ -calpain during isolation.

In summary identification of calpastatin in the mitochondrial intermembrane space, the same submitochondrial compartment as mitochondrial μ -calpain provides useful insight into questions related to the activation, substrates and function of mitochondrial μ -calpain. The results also suggest a possible mechanism of import of calpastatin to the mitochondrial intermembrane space and ascribe novel function to the XL domain of calpastatin.

Chapter six

Summary and Conclusions

“The essential matter of history is not what happened but what people thought or said about it.”- Frederic William Maitland

Calcium deregulation is central to the secondary neurodegeneration in traumatic neuronal injuries (Happel et al., 1981; Choi, 1988; Chesler et al., 1994; Saatman et al., 1996b). Mitochondria are one of the prime organelles to be involved in calcium regulation and deregulation (Rego and Oliveira, 2003; Bernardi and Rasola, 2007). Calpains, being calcium dependent proteases, are one of the key players in neuronal death resulting from calcium deregulation (Goll et al., 2003). Predominant ubiquitous isoforms of calpains present in the CNS are μ and m-calpain (Croall and DeMartino, 1991). m-Calpain caters to the physiological needs of the cell (Franco et al., 2004; Dutt et al., 2006) and is extra mitochondrial whereas μ -calpain has evidence for being tagged as a pathologic isoform (Siman et al., 1985; Roberts-Lewis et al., 1994; Azam et al., 2001; Cao et al., 2007) and is located in the mitochondrial intermembrane space (Badugu et al., 2008) as well as the cytosol. Thus, developing a better understanding of the mitochondrial μ -calpain could address the pathological consequences of calpain hyper-activation while simultaneously sparing normal cellular functions. **The primary objective of this dissertation was to gain insightful understanding into mitochondrial μ -calpain's function, with a focus on its putative role in the processing of apoptosis-inducing factor, and mitochondrial μ -calpain's regulation.**

Calpains are involved in wide variety of functions, both physiological and pathological (Goll et al., 2003). There are numerous known calpain substrates including cytoskeletal proteins, cell signaling proteins, and calcium regulatory proteins (Bever and Neumar, 2008). In previous studies of TBI, calpain inhibition demonstrated functional improvement (Saatman et al., 1996b) but without decrease in the cytoskeletal proteolysis, lesion volume or apoptosis (Saatman et al., 2000; Kupina et al., 2001), suggestive of some novel protein as a crucial substrate for calpain mediated neurodegeneration. There are number of mitochondrial proteins which are known to be calpain substrates such as AIF, BAX, BCL-XL, Bid, and NCX (Nakagawa and Yuan, 2000; Chen et al., 2001; Choi et al., 2001; Polster et al., 2005; Takano et al., 2005; Kar

et al., 2009). It is highly likely that these proteins could be processed by endogenous mitochondrial μ -calpain, whose function and substrates have not been addressed. Apoptosis-inducing factor (AIF) had very high probability of being a mitochondrial μ -calpain substrate because of its localization in the mitochondrial intermembrane space- the same intra-organelle compartment as mitochondrial μ -calpain (Garcia et al., 2005; Badugu et al., 2008), its requirement for processing at the N-terminus by an unknown protease to get detached from the inner mitochondrial membrane association (Otera et al., 2005), its essential role in inducing CICD (Cregan et al., 2002; Boujrad et al., 2007) which is documented to be involved in variety of neurodegenerative diseases (Cao et al., 2003; Plesnila et al., 2004; Liou et al., 2005; Yu et al., 2006a; Yu et al., 2006b; Wales et al., 2008) and evidence that AIF is cleaved by exogenous μ -calpain in isolated mitochondria (Polster et al., 2005) or in the cell culture models (Artus et al., 2006; Cao et al., 2007; Norberg et al., 2008; Wales et al., 2008; Mader et al., 2009). Identification of a protease which processes AIF and leads to CICD would help in inhibiting AIF release and translocation to the nucleus and also prevent the compromise in mitochondrial function due to AIF release. AIF being processed by endogenous mitochondrial μ -calpain seemed a very logical hypothesis. We thus studied the role of endogenous mitochondrial μ -calpain in AIF processing.

Chapter 3 of this dissertation provides evidence that mitochondrial μ -calpain is not involved in the processing of apoptosis-inducing factor. Atractyloside, an mPTP inducer, induced release of truncated AIF from the Percoll-enriched rat liver mitochondria following permeability transition pore formation. The release of Atr-induced tAIF occurred even in the presence of calpain inhibitors and mitochondrial μ -calpain immunoreactivity was difficult to detect in these mitochondria. This suggests that mitochondrial μ -calpain is not required for AIF processing and release. Inhibition of AIF release and processing following Atr incubation by MDL28170, demonstrated that Atr-induced AIF processing and release is mediated by a cysteine protease.

Besides calpains, two other cysteine proteases are caspases and cathepsins. Caspases are not localized in the mitochondria (van Loo et al., 2002a). Cysteine cathepsins B, L, and S activity is observed in the mitochondria, and they may be involved in AIF processing (Yuste et al., 2005; Chaitanya and Babu, 2008). Cathepsin inhibitor CA-074 inhibited Atr-induced AIF release, though not completely, indicating a positive role of cathepsins to a certain extent in Atr-induced AIF processing and release.

Cathepsin B is a lysosomal protease but its alternatively spliced variant lacking exon 2 and 3 is targeted to mitochondria (Muntener et al., 2004) and it is catalytically inactive (Baici et al., 2006). Moreover, cathepsin B knockout did not prevent AIF release induced by DNA damage (Moubarak et al., 2007).

Lack of AIF processing in the SH-SY5Y mitochondria despite endogenous mitochondrial μ -calpain activation suggests that endogenous mitochondrial μ -calpain activation is not sufficient for AIF processing. Possible explanations could be that the cleavage site of AIF is sterically hindered in such a way that it is not accessible to the endogenous mitochondrial μ -calpain or AIF and endogenous mitochondrial μ -calpain are located in separate mitochondrial intermembrane space compartments. We also observed that following mitochondrial solubilization AIF processing and endogenous mitochondrial μ -calpain activation are concomitant and both of the above mentioned events are susceptible to MDL28170. After mitochondrial solubilization steric hindrance and compartmentalization are lost, AIF may become accessible to the endogenous mitochondrial μ -calpain. MDL28170 susceptibility of AIF truncation in the Ca^{+2} paradigm, like Atr model, demonstrates that AIF processing is mediated by a cysteine protease. Calpains are the only known mitochondrial calcium dependent cysteine proteases. However there are several reports regarding the nature of calpains in the mitochondria besides μ -calpain. Calpain-10, another cysteine protease which is calcium dependent and sensitive to MDL28170, has also been reported to be present in the mitochondria (Arrington et al., 2006). A mitochondrial μ -like calpain is also reported to be associated with mitochondria (Ozaki et al., 2007). The possibility of any other mitochondrial calpain's involvement in AIF processing has not been ruled out. Overall it can be concluded that the activation of endogenous mitochondrial μ -calpain is neither sufficient nor required for the processing of AIF.

Evidence exists in literature for positive role of μ -calpain in AIF processing but that may be cytosolic μ -calpain, which has ability to translocate to mitochondria following cellular death signals (Kuboki et al., 1992; Goll et al., 2003). One paradox which arises with the above mentioned explanation is the ability of cytosolic μ -calpain to process AIF and the inability of mitochondrial μ -calpain to process AIF. There is lack of definitive evidence suggesting a difference between the nature, substrate specificity or structure of cytosolic or mitochondrial μ -calpain although recently Ozaki et al (Ozaki et al., 2007) demonstrated that mitochondrial calpain was stained with similar antibodies as μ -calpain

and was inhibited by calpeptin but there were some differences such as lower pH, higher sensitivity to chymostatin, later elution on chromatography and higher mobility in the zymogram showing that mitochondrial calpain may be different from μ -calpain, but this needs further investigation. It is also noteworthy that susceptibility of AIF to protease processing is also regulated by other factors. The protein scythe interacts with AIF and regulates its stability (Desmots et al., 2008). AIF susceptibility is also affected by the redox state of the cell. Normally AIF exists as NAD(P)H bound dimer capable of interacting with other proteins via a loop. This interaction with proteins protects the AIF loop from proteolysis and thus AIF helps in organizing cristae and maintaining good ETC. But a decline in the pool of NAD(P)H or inner membrane remodeling makes AIF a monomer due to which AIF loop loses attachment with proteins and becomes susceptible to the proteases. This cleaved AIF can translocate to nucleus and cause DNA damage (Churbanova and Sevrioukova, 2008). In nutshell it is fair enough to conclude that AIF release seems to be regulated by multiple factors (including multiple proteases/ pathways) such as the type, strength and duration of insult as well as the cell type and physiological state of the cell. Chapter 3 has shed light on the role of endogenous mitochondrial μ -calpain in AIF processing.

It is widely appreciated that there is difference in the nature of mitochondria from various organs in terms of their susceptibility to various insults, their respiratory capacities, their ion homeostasis capabilities and also their mechanism of mPTP induction (Hulbert et al., 2006; Shalbuyeva et al., 2006; Dubinsky, 2009). These differences may account for differences of tissue specific response to similar insults. Understanding the nature of these differences can account for some of the specific organ properties. We observed marked differences in the liver and brain mitochondria in tAIF release following Atr incubation. Following incubation with Atr both liver and brain mitochondria showed swelling indicating permeability transition pore formation, but only liver mitochondria showed robust release of tAIF whereas brain mitochondria did not show any release of tAIF. This suggests a fundamental difference in the mechanism of tAIF release in the two mitochondrial populations. Brain mitochondria differ from liver mitochondria in the behavior of the mPTP and its pharmacological regulation (Panov et al., 2007). Our results indicate that there could be a difference in ANT (adenine nucleotide translocase), the ligand of Atr, between two mitochondrial populations. ANT transports ATP/ADP across inner mitochondrial membrane and is an integral inner mitochondrial membrane protein (Klingenberg, 1980). Three genes of ANT have been

identified in humans (Cozens et al., 1989; Ku et al., 1990), three isoforms ANT 1, ANT 2 and ANT 3 are found in humans and only ANT 1 and ANT 2 are found in rodents (Brandolin et al., 1993; Belzacq et al., 2002). Highest ANT 1 mRNA proportions are found in terminally differentiated tissues like skeletal muscles and brain whereas higher ANT 2 mRNA is found in proliferative and regenerative tissues like liver, spleen and fibroblasts (Doerner et al., 1997). This varied level of two isoforms may be the underlying cause of differential response of two mitochondrial populations to the Atr-induced tAIF release. It also suggests that Atr may modulate mitochondrial bioenergetics differently in two mitochondria and hence renders differential release of tAIF. Relatively easier release of tAIF from liver mitochondria ties well with the fact that liver tissue being regenerable is more susceptible to cell death whereas brain tissue has a better and sturdy inbuilt mechanism to prevent the release of IMS cell death inducing proteins. tAIF release was observed with the outer mitochondrial membrane permeabilizing agent, alamethicin, in both brain and liver mitochondria. This can be explained by the presence of unanchored AIF in the mitochondrial IMS.

The studies in isolated mitochondria model system provided novel and insightful information about the relationship between AIF and endogenous mitochondrial μ -calpain but it has inherent limitation of not recapitulating *in situ* scenarios. Mitochondrial μ -calpain activation is demonstrated to be not sufficient for AIF processing in isolated mitochondria (Joshi et al., 2009) but this may not be true in the cell culture system due to involvement of various cytosolic factors. These factors may facilitate mitochondrial μ -calpain's accessibility to AIF and hence the relationship may be different. μ -Calpain is also present in the cytosol and has the ability to translocate to the membranes. There is a possibility of cytosolic μ -calpain processing AIF. It is also known that tAIF is released from mitochondria and subsequently translocates to the nucleus and induces large scale DNA fragmentation (50 kB). The role of calpains in AIF nuclear translocation also needs to be addressed. Chapter 4 of the dissertation was an effort to address the above mentioned issues. This part of dissertation explored the role of total cellular calpain in AIF processing and distribution to the nucleus in cell culture model system. NMDA excitotoxicity induced calpain activation, no AIF processing and showed perinuclear AIF immunostaining. This demonstrates that calpain activation does not necessarily induce AIF processing and AIF translocation to the nucleus. We observed that A23187 induced calpain activation and no AIF processing but still showed AIF nuclear translocation, which occurred in the presence of MDL28170. This suggests that AIF translocation to

the nucleus can occur independent of calpain activity and even without processing. This is in agreement with the recent observation that AIF translocates to the nucleus in Calpain 4^{-/-} mice, which are functional calpain knockouts, following N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) or NMDA treatment (Wang et al., 2009b). It is also in agreement with the observation of chapter 3 where Atr treated rat liver isolated mitochondria released tAIF even in the absence of μ -calpain activation.

There is relatively little information regarding the regulation of mitochondrial μ -calpain. An activator of mitochondrial μ -calpain has been identified – Acyl-CoA binding protein (ACBP) (Melloni et al., 2000; Van Loo et al., 2002b; Shulga and Pastorino, 2006). But no inhibitory component has been yet identified. Initially it was believed that mitochondrial localization of μ -calpain would segregate the enzyme from its inhibitor-calpastatin, which was thought to be predominantly cytosolic (Taylor et al., 1991; Kumamoto et al., 1995), and also place μ -calpain in the vicinity of calcium released following mPTP opening, making its activation easier. Surprisingly, a large amount of calcium (mM) was required for activating mitochondrial μ -calpain in our experiments (Joshi et al., 2009). Chapter 5 of the dissertation thus ventured to provide novel insight into the regulation of mitochondrial μ -calpain. These studies report, for the first time, the association of calpastatin to the Percoll-enriched mitochondrial fraction of the human SH-SY5Y cells and rat cerebral cortex. Further we demonstrate by proteinase K protection assay that calpastatin is inside the mitochondria and by mitochondrial subfractionation that the calpastatin is localized in the mitochondrial intermembrane space. This localization is same as that of mitochondrial μ -calpain and may explain why we need high concentrations (mM) of calcium for activating mitochondrial μ -calpain. One possible explanation for the lack of involvement of mitochondrial μ -calpain in AIF processing following mPTP could be the presence of mitochondrial calpastatin.

Calpastatin is nuclear encoded, synthesized in the cytosol and has to be imported into the mitochondria. The mechanism of calpastatin import into the mitochondria was addressed by demonstrating that the N-terminal first 30 amino acids of calpastatin preferentially localize to the mitochondria in the cells transfected with 1-30XLDCAST-pWPXL. This demonstrates that the N-terminal sequence of the XL domain of calpastatin is a mitochondrial targeting sequence. There are variety of isoforms of calpastatin with or without XL domain. Since XL domain has the mitochondrial targeting sequence, it is possible that XL domain containing calpastatin is exclusively

mitochondrial. The differential expression of XL domain calpastatin isoform may be a means to modulate the activity of mitochondrial calpains.

The functional significance for the presence of μ -calpain in the mitochondrial IMS remains to be determined. Recently NCX, a major means of extruding calcium out of the mitochondria, was identified as a mitochondrial μ -calpain substrate (Kar et al., 2009). Calpains are involved in cell adhesion, motility, and dynamics similarly mitochondrial μ -calpain may be involved in the mitochondrial dynamics and regulating mitochondrial morphology. The components of electron transport chain or mPTP may also be substrates of mitochondrial μ -calpain. The presence of calpastatin has its own implication and further complicates the scene to understand how mitochondrial calpains are activated in the mitochondria. Calpastatin binds to EF hands on both the domain IV and domain VI for inhibiting calpains (Moldoveanu et al., 2008). Mitochondrial calpastatin will negatively regulate the activity of mitochondrial calpains, but calpain-10, which is mitochondrial and is reported to be involved in mPTP formation and compromising complex I activity of ETC by proteolysing subunits NDUFV2 and ND6 (Arrington et al., 2006), may not be affected by mitochondrial calpastatin as it lacks the domain IV (Bevers and Neumar, 2008). Further work needs to be done to understand how mitochondrial calpastatin interacts and regulates mitochondrial μ -calpain, the fraction of total calpastatin in the mitochondria - whether XL domain containing calpastatin is exclusively mitochondrial, and if there are any changes in the relative expression level of various calpastatin isoforms, particularly XL domain containing calpastatin during injury, development or normal physiology.

Finally this dissertation provides novel insights into the mechanism of activation, function, with regard to AIF processing, and regulation of the endogenous mitochondrial μ -calpain. We have refuted the hypothesis that localization of μ -calpain in the mitochondria would make its activation fast, reliable, and easier by proximity to the activator – calcium and segregation from the inhibitor – calpastatin. We demonstrate that the activation of endogenous mitochondrial μ -calpain requires relatively high calcium concentrations. We describe the function of endogenous mitochondrial μ -calpain with regard to AIF processing. We provide the first evidence that the endogenous mitochondrial μ -calpain is not involved in the processing of AIF by showing that the endogenous mitochondrial μ -calpain activation is neither sufficient nor required for the processing of AIF. We also demonstrate that total cellular calpain activation is not

sufficient for AIF processing, AIF processing is independent of calpain activation suggesting involvement of multiple protease pathways, and AIF can translocate to the nucleus even as full length mature 62 kD unprocessed form. Novel findings of this dissertation also include the localization of the calpain inhibitor-calpastatin to the mitochondrial IMS and further unfolding the mechanism of import of calpastatin to the mitochondria by determining a mitochondrial targeting sequence in the N-terminus of the XL domain of calpastatin. Although these studies helped to answer several questions regarding the substrate and regulation of mitochondrial μ -calpain, they raise many more questions and much remains to be learned.

Appendix

Glossary of Terms

%	- Percentage	D	- After differential fraction
$\Delta\Psi$	- Membrane potential	Da	- Dalton
μg	- Microgram	Diablo	- Direct IAP binding protein of low PI
μM	- Micromolar	DIV	- Days <i>in vitro</i>
$^{\circ}\text{C}$	- Degree celsius	DLAR	- Division of lab animal resources
α	- Alpha	DNA	- Deoxyribonucleic acid
μ	- Mu or Micro	e.g.	- Example
β	- Beta	E18	- Embryonic day 18 pups
aa	- Amino acid	E9	- Embryonic day 9 pups
AAALC	- American association for the accreditation of laboratory animal care	EDTA	- Ethylene diamine tetra acetic acid
ACBP	- Acyl-CoA binding protein	EGTA	- Ethylene glycol tetra acetic acid
ADP	- Adenosine diphosphate	EndoG	- Endonuclease G
AIF	- Apoptosis-inducing factor	ER	- Endoplasmic reticulum
Alm	- Alamethicin	ETC	- Electron transport chain
ANOVA	- Analysis of variance	FAD	- Flavin adenine dinucleotide
ANT	- Adenine nucleotide translocase	Fig	- Figure
Asn	- Asparagine	g	- g-force
Arg	- Arginine	GFP	- Green fluorescent protein
ATP	- Adenosine triphosphate	H	- Homogenate
Atr	- Atractyloside	h	- Hour
BBB	- Blood brain barrier	HBSS	- Hank's buffered salts solution
BCA	- Bicinchoninic acid	HEL	- Human erythroleukemia cells
BSA	- Bovine serum albumin	HEPES	- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
CA	- CA-074	HGF	- Hepatocyte growth factor
Ca^{2+}	- Calcium	His	- Histidine
CaCl_2	- Calcium chloride	Hq	- Harlequin
Calpain 4 -/-	- Calpain 4 knock out	HSP70	- Heat shock protein 70
CAP	- Calpain activating protein	IAP	- Inhibitor of apoptosis protein
CAST	- Calpastatin	ICC	- Immunocytochemistry
cat. no.	- Catalogue number	i.e.	- That is
CDC	- Center for disease control	IMM	- Inner mitochondrial membrane
CICD	- Caspase-independent cell death	IMS	- Intermembrane space
CNS	- Central nervous system	IP3R	- Inositol triphosphate receptor
Cnt	- Control	kB	- Kilobase
CO_2	- Carbon dioxide		
CRMP-3	- Collapsin response mediator protein		
CsA	- Cyclosporine A		
CypD	- Cyclophilin D		
Cys	- Cysteine		
CytC	- Cytochrome C		

KCl	- Potassium chloride	PAR	- Poly-ADP ribose
kD	- Kilodalton	PARG	- Poly-ADP ribose polymerase glycohydrolase
KH ₂ PO ₄	- Potassium dihydrogen phosphate	PARP	- Poly-ADP ribose polymerase
KOH	- Potassium hydroxide	PBR	- Peripheral benzodiazepine receptor
Lys	- Lysine	PBS	- Phosphate buffered saline
M	- Mitochondrial fraction	PCR	- Polymerase chain reaction
MCA	- Middle cerebral artery	PD	- PD150606
MDL	- MDL28170	PK	- Proteinase K
MEM	- Minimum essential media	PKA	- Protein kinase A
mg	- Milligram	PKC	- Protein kinase C
Mg ²⁺	- Magnesium	PMSF	- Phenyl methane sulfonyl fluoride
MgCl ₂	- Magnesium chloride	ROS	- Reactive oxygen species
mHSP70	- Mitochondrial HSP70	rpm	- Revolutions per minute
MIB	- Mitochondrial isolation buffer	s	- Seconds
min	- Minutes	SDS	- Sodium dodecyl sulfate
ml	- Milliliter	SEM	- Standard error of mean
MLS	- Mitochondrial localization signal	SERCA	- Sarcoplasmic endoplasmic reticulum calcium ATPase
MTS	- Mitochondrial targeting sequence	siRNA	- Small interfering RNA
mM	- Millimolar	Smac	- Second mitochondrial activator of caspases
MNNG	- N-Methyl-N'-Nitro-N-Nitrosoguanidine	tAIF	- Truncated AIF
MP	- Mitoplast fraction	TBI	- Traumatic brain injury
mPTP	- Mitochondrial permeability transition pore	tBid	- Truncated Bid
mtDNA	- Mitochondrial DNA	TBS	- Tris buffered saline
mRNA	- Messenger RNA	TIM 22	- Translocase of inner membrane 22
RNA	- Ribonucleic acid	TIM 23	- Translocase of inner membrane 23
NA	- Not applicable	TNF	- Tumor necrosis factor
NAD(P)H	- Nicotinamide adenine dinucleotide phosphate	TOM	- Translocase of outer membrane
NADH	- Nicotinamide adenine dinucleotide	TOB	- Topogenesis of mitochondrial outer-membrane β -barrel proteins
NCX	- Sodium calcium exchanger	tSCI	- Traumatic spinal cord injury
NFKB	- Nuclear factor kappa b	TTBS	- Tween-Tris buffered saline
N	- Amino terminus	Tx	- Triton X
NLS	- Nuclear localization signal	uM	- Micromolar
MPP+	- 1-methyl-4-phenylpyridinium	VDAC	- Voltage dependent anion channel
nM	- Nanomolar	Zn ²⁺	- Zinc
NMDA	- N-methyl-D-aspartic acid		
NO	- Nitric oxide		
NS	- Non significant		
OMM	- Outer mitochondrial membrane		
p53	- Protein 53		
PAGE	- Polyacrylamide gel electrophoresis		

References

- Adachi Y, Ishida-Takahashi A, Takahashi C, Takano E, Murachi T, Hatanaka M (1991) Phosphorylation and subcellular distribution of calpastatin in human hematopoietic system cells. *J Biol Chem* 266:3968-3972.
- Anderson S, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJ, Staden R, Young IG (1981) Sequence and organization of the human mitochondrial genome. *Nature* 290:457-465.
- Andrabi SA, Kim NS, Yu SW, Wang H, Koh DW, Sasaki M, Klaus JA, Otsuka T, Zhang Z, Koehler RC, Hurn PD, Poirier GG, Dawson VL, Dawson TM (2006) Poly(ADP-ribose) (PAR) polymer is a death signal. *Proc Natl Acad Sci U S A* 103:18308-18313.
- Arai K, Lee SR, van Leyen K, Kurose H, Lo EH (2004) Involvement of ERK MAP kinase in endoplasmic reticulum stress in SH-SY5Y human neuroblastoma cells. *J Neurochem* 89:232-239.
- Armstrong JS (2006) Mitochondrial membrane permeabilization: the sine qua non for cell death. *Bioessays* 28:253-260.
- Arrington DD, Van Vleet TR, Schnellmann RG (2006) Calpain 10: a mitochondrial calpain and its role in calcium-induced mitochondrial dysfunction. *Am J Physiol Cell Physiol* 291:C1159-1171.
- Arthur JS, Elce JS, Hegadorn C, Williams K, Greer PA (2000) Disruption of the murine calpain small subunit gene, *Capn4*: calpain is essential for embryonic development but not for cell growth and division. *Mol Cell Biol* 20:4474-4481.
- Artus C, Maquarre E, Moubarak RS, Delettre C, Jasmin C, Susin SA, Robert-Lezennes J (2006) CD44 ligation induces caspase-independent cell death via a novel calpain/AIF pathway in human erythroleukemia cells. *Oncogene* 25:5741-5751.
- Austin JW, Fehlings MG (2008) Molecular mechanisms of Fas-mediated cell death in oligodendrocytes. *J Neurotrauma* 25:411-426.
- Averna M, de Tullio R, Passalacqua M, Salamino F, Pontremoli S, Melloni E (2001) Changes in intracellular calpastatin localization are mediated by reversible phosphorylation. *Biochem J* 354:25-30.
- Azam M, Andrabi SS, Sahr KE, Kamath L, Kuliopulos A, Chishti AH (2001) Disruption of the mouse mu-calpain gene reveals an essential role in platelet function. *Mol Cell Biol* 21:2213-2220.
- Badugu R, Garcia M, Bondada V, Joshi A, Geddes JW (2008) N terminus of calpain 1 is a mitochondrial targeting sequence. *J Biol Chem* 283:3409-3417.
- Baici A, Muntener K, Willmann A, Zwicky R (2006) Regulation of human cathepsin B by alternative mRNA splicing: homeostasis, fatal errors and cell death. *Biol Chem* 387:1017-1021.
- Baines CP, Kaiser RA, Sheiko T, Craigen WJ, Molkentin JD (2007) Voltage-dependent anion channels are dispensable for mitochondrial-dependent cell death. *Nat Cell Biol* 9:550-555.
- Baines CP, Kaiser RA, Purcell NH, Blair NS, Osinska H, Hambleton MA, Brunskill EW, Sayen MR, Gottlieb RA, Dorn GW, Robbins J, Molkentin JD (2005) Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. *Nature* 434:658-662.
- Banik NL, Matzelle D, Gantt-Wilford G, Hogan EL (1997) Role of calpain and its inhibitors in tissue degeneration and neuroprotection in spinal cord injury. *Ann N Y Acad Sci* 825:120-127.

- Banik NL, Shields DC, Ray S, Davis B, Matzelle D, Wilford G, Hogan EL (1998) Role of calpain in spinal cord injury: effects of calpain and free radical inhibitors. *Ann N Y Acad Sci* 844:131-137.
- Bannai H, Tamada Y, Maruyama O, Nakai K, Miyano S (2002) Extensive feature detection of N-terminal protein sorting signals. *Bioinformatics* 18:298-305.
- Bano D, Young KW, Guerin CJ, Lefevre R, Rothwell NJ, Naldini L, Rizzuto R, Carafoli E, Nicotera P (2005) Cleavage of the plasma membrane $\text{Na}^+/\text{Ca}^{2+}$ exchanger in excitotoxicity. *Cell* 120:275-285.
- Barnoy S, Zipser Y, Glaser T, Grimberg Y, Kosower NS (1999) Association of calpain (Ca^{2+} -dependent thiol protease) with its endogenous inhibitor calpastatin in myoblasts. *J Cell Biochem* 74:522-531.
- Basso E, Fante L, Fowlkes J, Petronilli V, Forte MA, Bernardi P (2005) Properties of the permeability transition pore in mitochondria devoid of Cyclophilin D. *J Biol Chem* 280:18558-18561.
- Bednarski E, Vanderklish P, Gall C, Saido TC, Bahr BA, Lynch G (1995) Translational suppression of calpain I reduces NMDA-induced spectrin proteolysis and pathophysiology in cultured hippocampal slices. *Brain Res* 694:147-157.
- Beer DG, Hjelle JJ, Petersen DR, Malkinson AM (1982) Calcium-activated proteolytic activity in rat liver mitochondria. *Biochem Biophys Res Commun* 109:1276-1283.
- Belzacq AS, Vieira HL, Kroemer G, Brenner C (2002) The adenine nucleotide translocator in apoptosis. *Biochimie* 84:167-176.
- Bernardi P (1992) Modulation of the mitochondrial cyclosporin A-sensitive permeability transition pore by the proton electrochemical gradient. Evidence that the pore can be opened by membrane depolarization. *J Biol Chem* 267:8834-8839.
- Bernardi P (1996) The permeability transition pore. Control points of a cyclosporin A-sensitive mitochondrial channel involved in cell death. *Biochim Biophys Acta* 1275:5-9.
- Bernardi P, Rasola A (2007) Calcium and cell death: the mitochondrial connection. *Subcell Biochem* 45:481-506.
- Bernardi P, Colonna R, Costantini P, Eriksson O, Fontaine E, Ichas F, Massari S, Nicolli A, Petronilli V, Scorrano L (1998) The mitochondrial permeability transition. *Biofactors* 8:273-281.
- Beyers MB, Neumar RW (2008) Mechanistic role of calpains in postischemic neurodegeneration. *J Cereb Blood Flow Metab* 28:655-673.
- Bi R, Bi X, Baudry M (1998) Phosphorylation regulates calpain-mediated truncation of glutamate ionotropic receptors. *Brain Res* 797:154-158.
- Blomgren K, Hallin U, Andersson AL, Puka-Sundvall M, Bahr BA, McRae A, Saido TC, Kawashima S, Hagberg H (1999) Calpastatin is up-regulated in response to hypoxia and is a suicide substrate to calpain after neonatal cerebral hypoxia-ischemia. *J Biol Chem* 274:14046-14052.
- Boujrad H, Gubkina O, Robert N, Krantic S, Susin SA (2007) AIF-mediated programmed necrosis: a highly regulated way to die. *Cell Cycle* 6:2612-2619.
- Bramlett HM, Dietrich WD (2004) Pathophysiology of cerebral ischemia and brain trauma: similarities and differences. *J Cereb Blood Flow Metab* 24:133-150.
- Brandolin G, Le Saux A, Trezeguet V, Lauquin GJ, Vignais PV (1993) Chemical, immunological, enzymatic, and genetic approaches to studying the arrangement of the peptide chain of the ADP/ATP carrier in the mitochondrial membrane. *J Bioenerg Biomembr* 25:459-472.
- Brewer GJ, Torricelli JR, Evege EK, Price PJ (1993) Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. *J Neurosci Res* 35:567-576.

- Broekemeier KM, Dempsey ME, Pfeiffer DR (1989) Cyclosporin A is a potent inhibitor of the inner membrane permeability transition in liver mitochondria. *J Biol Chem* 264:7826-7830.
- Brookes PS, Yoon Y, Robotham JL, Anders MW, Sheu SS (2004) Calcium, ATP, and ROS: a mitochondrial love-hate triangle. *Am J Physiol Cell Physiol* 287:C817-833.
- Brorson JR, Marcuccilli CJ, Miller RJ (1995) Delayed antagonism of calpain reduces excitotoxicity in cultured neurons. *Stroke* 26:1259-1266; discussion 1267.
- Brown D, Yu BD, Joza N, Benit P, Meneses J, Firpo M, Rustin P, Penninger JM, Martin GR (2006a) Loss of Aif function causes cell death in the mouse embryo, but the temporal progression of patterning is normal. *Proc Natl Acad Sci U S A* 103:9918-9923.
- Brown MR, Sullivan PG, Geddes JW (2006b) Synaptic mitochondria are more susceptible to Ca²⁺ overload than nonsynaptic mitochondria. *J Biol Chem* 281:11658-11668.
- Brustis JJ, Elamrani N, Balcerzak D, Safwate A, Soriano M, Poussard S, Cottin P, Ducastaing A (1994) Rat myoblast fusion requires exteriorized m-calpain activity. *Eur J Cell Biol* 64:320-327.
- Brustovetsky N, Brustovetsky T, Jemmerson R, Dubinsky JM (2002) Calcium-induced cytochrome c release from CNS mitochondria is associated with the permeability transition and rupture of the outer membrane. *J Neurochem* 80:207-218.
- Buki A, Siman R, Trojanowski JQ, Povlishock JT (1999) The role of calpain-mediated spectrin proteolysis in traumatically induced axonal injury. *J Neuropathol Exp Neurol* 58:365-375.
- Buki A, Farkas O, Doczi T, Povlishock JT (2003) Preinjury administration of the calpain inhibitor MDL-28170 attenuates traumatically induced axonal injury. *J Neurotrauma* 20:261-268.
- Cande C, Vahsen N, Kouranti I, Schmitt E, Daugas E, Spahr C, Luban J, Kroemer RT, Giordanetto F, Garrido C, Penninger JM, Kroemer G (2004) AIF and cyclophilin A cooperate in apoptosis-associated chromatinolysis. *Oncogene* 23:1514-1521.
- Cao G, Clark RS, Pei W, Yin W, Zhang F, Sun FY, Graham SH, Chen J (2003) Translocation of apoptosis-inducing factor in vulnerable neurons after transient cerebral ischemia and in neuronal cultures after oxygen-glucose deprivation. *J Cereb Blood Flow Metab* 23:1137-1150.
- Cao G, Xing J, Xiao X, Liou AK, Gao Y, Yin XM, Clark RS, Graham SH, Chen J (2007) Critical role of calpain I in mitochondrial release of apoptosis-inducing factor in ischemic neuronal injury. *J Neurosci* 27:9278-9293.
- Carragher NO (2006) Calpain inhibition: a therapeutic strategy targeting multiple disease states. *Curr Pharm Des* 12:615-638.
- Carragher NO, Westhoff MA, Riley D, Potter DA, Dutt P, Elce JS, Greer PA, Frame MC (2002) v-Src-induced modulation of the calpain-calpastatin proteolytic system regulates transformation. *Mol Cell Biol* 22:257-269.
- CDC A (2006) National center for injury prevention and control, CDC TBI and tSCI data fact sheet. In.
- Chaitanya GV, Babu PP (2008) Multiple apoptogenic proteins are involved in the nuclear translocation of Apoptosis Inducing Factor during transient focal cerebral ischemia in rat. *Brain Res* 1246:178-190.
- Chatterjee S, Gu ZQ, Dunn D, Tao M, Josef K, Tripathy R, Bihovsky R, Senadhi SE, O'Kane TM, McKenna BA, Mallya S, Ator MA, Bozyczko-Coyne D, Siman R, Mallamo JP (1998) D-amino acid containing, high-affinity inhibitors of recombinant human calpain I. *J Med Chem* 41:2663-2666.

- Chen L, Willis SN, Wei A, Smith BJ, Fletcher JI, Hinds MG, Colman PM, Day CL, Adams JM, Huang DC (2005) Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. *Mol Cell* 17:393-403.
- Chen M, He H, Zhan S, Krajewski S, Reed JC, Gottlieb RA (2001) Bid is cleaved by calpain to an active fragment in vitro and during myocardial ischemia/reperfusion. *J Biol Chem* 276:30724-30728.
- Chesler M, Young W, Hassan AZ, Sakatani K, Moriya T (1994) Elevation and clearance of extracellular K⁺ following graded contusion of the rat spinal cord. *Exp Neurol* 125:93-98.
- Cheung EC, Melanson-Drapeau L, Cregan SP, Vanderluit JL, Ferguson KL, McIntosh WC, Park DS, Bennett SA, Slack RS (2005) Apoptosis-inducing factor is a key factor in neuronal cell death propagated by BAX-dependent and BAX-independent mechanisms. *J Neurosci* 25:1324-1334.
- Cheung EC, Joza N, Steenaart NA, McClellan KA, Neuspiel M, McNamara S, MacLaurin JG, Rippstein P, Park DS, Shore GC, McBride HM, Penninger JM, Slack RS (2006) Dissociating the dual roles of apoptosis-inducing factor in maintaining mitochondrial structure and apoptosis. *EMBO J* 25:4061-4073.
- Choi DW (1988) Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage. *Trends Neurosci* 11:465-469.
- Choi WS, Lee EH, Chung CW, Jung YK, Jin BK, Kim SU, Oh TH, Saido TC, Oh YJ (2001) Cleavage of Bax is mediated by caspase-dependent or -independent calpain activation in dopaminergic neuronal cells: protective role of Bcl-2. *J Neurochem* 77:1531-1541.
- Churbanova IY, Sevrioukova IF (2008) Redox-dependent changes in molecular properties of mitochondrial apoptosis-inducing factor. *J Biol Chem* 283:5622-5631.
- Claros MG, Vincens P (1996) Computational method to predict mitochondrially imported proteins and their targeting sequences. *Eur J Biochem* 241:779-786.
- Cong J, Goll DE, Peterson AM, Kapprell HP (1989) The role of autolysis in activity of the Ca²⁺-dependent proteinases (μ -calpain and m-calpain). *J Biol Chem* 264:10096-10103.
- Cong M, Thompson VF, Goll DE, Antin PB (1998) The bovine calpastatin gene promoter and a new N-terminal region of the protein are targets for cAMP-dependent protein kinase activity. *J Biol Chem* 273:660-666.
- Cottin P, Vidalenc PL, Ducastaing A (1981) Ca²⁺-dependent association between a Ca²⁺-activated neutral proteinase (CaANP) and its specific inhibitor. *FEBS Lett* 136:221-224.
- Cozens AL, Runswick MJ, Walker JE (1989) DNA sequences of two expressed nuclear genes for human mitochondrial ADP/ATP translocase. *J Mol Biol* 206:261-280.
- Crawford C, Brown NR, Willis AC (1993) Studies of the active site of m-calpain and the interaction with calpastatin. *Biochem J* 296 (Pt 1):135-142.
- Cregan SP, Dawson VL, Slack RS (2004) Role of AIF in caspase-dependent and caspase-independent cell death. *Oncogene* 23:2785-2796.
- Cregan SP, Fortin A, MacLaurin JG, Callaghan SM, Cecconi F, Yu SW, Dawson TM, Dawson VL, Park DS, Kroemer G, Slack RS (2002) Apoptosis-inducing factor is involved in the regulation of caspase-independent neuronal cell death. *J Cell Biol* 158:507-517.
- Croall DE, DeMartino GN (1991) Calcium-activated neutral protease (calpain) system: structure, function, and regulation. *Physiol Rev* 71:813-847.
- Crocker SJ, Smith PD, Jackson-Lewis V, Lamba WR, Hayley SP, Grimm E, Callaghan SM, Slack RS, Melloni E, Przedborski S, Robertson GS, Anisman H, Merali Z,

- Park DS (2003) Inhibition of calpains prevents neuronal and behavioral deficits in an MPTP mouse model of Parkinson's disease. *J Neurosci* 23:4081-4091.
- Crompton M, Ellinger H, Costi A (1988) Inhibition by cyclosporin A of a Ca^{2+} -dependent pore in heart mitochondria activated by inorganic phosphate and oxidative stress. *Biochem J* 255:357-360.
- Crompton M, Virji S, Ward JM (1998) Cyclophilin-D binds strongly to complexes of the voltage-dependent anion channel and the adenine nucleotide translocase to form the permeability transition pore. *Eur J Biochem* 258:729-735.
- Cuerrier D, Moldoveanu T, Davies PL (2005) Determination of peptide substrate specificity for mu-calpain by a peptide library-based approach: the importance of primed side interactions. *J Biol Chem* 280:40632-40641.
- Czogalla A, Sikorski AF (2005) Spectrin and calpain: a 'target' and a 'sniper' in the pathology of neuronal cells. *Cell Mol Life Sci* 62:1913-1924.
- Dahmer MK (2005) Caspases-2, -3, and -7 are involved in thapsigargin-induced apoptosis of SH-SY5Y neuroblastoma cells. *J Neurosci Res* 80:576-583.
- Daniel PT, Schulze-Osthoff K, Belka C, Guner D (2003) Guardians of cell death: the Bcl-2 family proteins. *Essays Biochem* 39:73-88.
- Daugas E, Nochy D, Ravagnan L, Loeffler M, Susin SA, Zamzami N, Kroemer G (2000a) Apoptosis-inducing factor (AIF): a ubiquitous mitochondrial oxidoreductase involved in apoptosis. *FEBS Lett* 476:118-123.
- Daugas E, Susin SA, Zamzami N, Ferri KF, Irinopoulou T, Larochette N, Prevost MC, Leber B, Andrews D, Penninger J, Kroemer G (2000b) Mitochondrio-nuclear translocation of AIF in apoptosis and necrosis. *Faseb J* 14:729-739.
- Dayton WR (1982) Comparison of low- and high-calcium-requiring forms of the calcium-activated protease with their autocatalytic breakdown products. *Biochim Biophys Acta* 709:166-172.
- Dayton WR, Reville WJ, Goll DE, Stromer MH (1976) A Ca^{2+} -activated protease possibly involved in myofibrillar protein turnover. Partial characterization of the purified enzyme. *Biochemistry* 15:2159-2167.
- De Giorgi F, Lartigue L, Ichas F (2000) Electrical coupling and plasticity of the mitochondrial network. *Cell Calcium* 28:365-370.
- De Jongh KS, Colvin AA, Wang KK, Catterall WA (1994) Differential proteolysis of the full-length form of the L-type calcium channel $\alpha 1$ subunit by calpain. *J Neurochem* 63:1558-1564.
- De Tullio R, Sparatore B, Salamino F, Melloni E, Pontremoli S (1998) Rat brain contains multiple mRNAs for calpastatin. *FEBS Lett* 422:113-117.
- De Tullio R, Aversa M, Stifanese R, Parr T, Bardsley RG, Pontremoli S, Melloni E (2007) Multiple rat brain calpastatin forms are produced by distinct starting points and alternative splicing of the N-terminal exons. *Arch Biochem Biophys* 465:148-156.
- Delettre C, Yuste VJ, Moubarak RS, Bras M, Robert N, Susin SA (2006a) Identification and characterization of AIFsh2, a mitochondrial apoptosis-inducing factor (AIF) isoform with NADH oxidase activity. *J Biol Chem* 281:18507-18518.
- Delettre C, Yuste VJ, Moubarak RS, Bras M, Lesbordes-Brion JC, Petres S, Bellalou J, Susin SA (2006b) AIFsh, a novel apoptosis-inducing factor (AIF) pro-apoptotic isoform with potential pathological relevance in human cancer. *J Biol Chem* 281:6413-6427.
- DeMartino GN, Wachendorfer R, McGuire MJ, Croall DE (1988) Proteolysis of the protein inhibitor of calcium-dependent proteases produces lower molecular weight fragments that retain inhibitory activity. *Arch Biochem Biophys* 262:189-198.

- Desmots F, Russell HR, Michel D, McKinnon PJ (2008) Scythe regulates apoptosis-inducing factor stability during endoplasmic reticulum stress-induced apoptosis. *J Biol Chem* 283:3264-3271.
- Doerner A, Pauschinger M, Badorff A, Noutsias M, Giessen S, Schulze K, Bilger J, Rauch U, Schultheiss HP (1997) Tissue-specific transcription pattern of the adenine nucleotide translocase isoforms in humans. *FEBS Lett* 414:258-262.
- Donkor IO (2000) A survey of calpain inhibitors. *Curr Med Chem* 7:1171-1188.
- Du C, Fang M, Li Y, Li L, Wang X (2000) Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell* 102:33-42.
- Dubinsky JM (2009) Heterogeneity of nervous system mitochondria: Location, location, location! *Exp Neurol*.
- Dutt P, Croall DE, Arthur SC, De Veyra T, Williams K, Elce JS, Greer PA (2006) m-Calpain is required for preimplantation embryonic development in mice. *BMC Dev Biol* 6:3.
- Edmunds T, Nagainis PA, Sathe SK, Thompson VF, Goll DE (1991) Comparison of the autolyzed and unautolyzed forms of mu- and m-calpain from bovine skeletal muscle. *Biochim Biophys Acta* 1077:197-208.
- Ekert PG, Read SH, Silke J, Marsden VS, Kaufmann H, Hawkins CJ, Gerl R, Kumar S, Vaux DL (2004) Apaf-1 and caspase-9 accelerate apoptosis, but do not determine whether factor-deprived or drug-treated cells die. *J Cell Biol* 165:835-842.
- Elce JS, Hegadorn C, Arthur JS (1997) Autolysis, Ca²⁺ requirement, and heterodimer stability in m-calpain. *J Biol Chem* 272:11268-11275.
- Emanuelsson O, Brunak S, von Heijne G, Nielsen H (2007) Locating proteins in the cell using TargetP, SignalP and related tools. *Nat Protoc* 2:953-971.
- Emori Y, Kawasaki H, Imajoh S, Imahori K, Suzuki K (1987) Endogenous inhibitor for calcium-dependent cysteine protease contains four internal repeats that could be responsible for its multiple reactive sites. *Proc Natl Acad Sci U S A* 84:3590-3594.
- Eskes R, Desagher S, Antonsson B, Martinou JC (2000) Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. *Mol Cell Biol* 20:929-935.
- Farooque M, Hillered L, Holtz A, Olsson Y (1996) Changes of extracellular levels of amino acids after graded compression trauma to the spinal cord: an experimental study in the rat using microdialysis. *J Neurotrauma* 13:537-548.
- Ferrand-Drake M, Zhu C, Gido G, Hansen AJ, Karlsson JO, Bahr BA, Zamzami N, Kroemer G, Chan PH, Wieloch T, Blomgren K (2003) Cyclosporin A prevents calpain activation despite increased intracellular calcium concentrations, as well as translocation of apoptosis-inducing factor, cytochrome c and caspase-3 activation in neurons exposed to transient hypoglycemia. *J Neurochem* 85:1431-1442.
- Figueiredo-Pereira ME, Banik N, Wilk S (1994) Comparison of the effect of calpain inhibitors on two extralysosomal proteinases: the multicatalytic proteinase complex and m-calpain. *J Neurochem* 62:1989-1994.
- Fonnum F (1984) Glutamate: a neurotransmitter in mammalian brain. *J Neurochem* 42:1-11.
- Fournier N, Ducet G, Crevat A (1987) Action of cyclosporine on mitochondrial calcium fluxes. *J Bioenerg Biomembr* 19:297-303.

- Franco SJ, Rodgers MA, Perrin BJ, Han J, Bennin DA, Critchley DR, Huttenlocher A (2004) Calpain-mediated proteolysis of talin regulates adhesion dynamics. *Nat Cell Biol* 6:977-983.
- Frey TG, Mannella CA (2000) The internal structure of mitochondria. *Trends Biochem Sci* 25:319-324.
- Frey TG, Renken CW, Perkins GA (2002) Insight into mitochondrial structure and function from electron tomography. *Biochim Biophys Acta* 1555:196-203.
- Friedrich P, Bozoky Z (2005) Digestive versus regulatory proteases: on calpain action in vivo. *Biol Chem* 386:609-612.
- Gafni J, Ellerby LM (2002) Calpain activation in Huntington's disease. *J Neurosci* 22:4842-4849.
- Gao G, Dou QP (2000) N-terminal cleavage of bax by calpain generates a potent proapoptotic 18-kDa fragment that promotes bcl-2-independent cytochrome C release and apoptotic cell death. *J Cell Biochem* 80:53-72.
- Garcia M, Bondada V, Geddes JW (2005) Mitochondrial localization of mu-calpain. *Biochem Biophys Res Commun* 338:1241-1247.
- Garesse R, Vallejo CG (2001) Animal mitochondrial biogenesis and function: a regulatory cross-talk between two genomes. *Gene* 263:1-16.
- Gerencsér AA, Adam-Vizi V (2005) Mitochondrial Ca²⁺ dynamics reveals limited intramitochondrial Ca²⁺ diffusion. *Biophys J* 88:698-714.
- Gil-Parrado S, Popp O, Knoch TA, Zahler S, Bestvater F, Felgentrager M, Holloschi A, Fernandez-Montalvan A, Auerswald EA, Fritz H, Fuentes-Prior P, Machleidt W, Spiess E (2003) Subcellular localization and in vivo subunit interactions of ubiquitous mu-calpain. *J Biol Chem* 278:16336-16346.
- Glading A, Chang P, Lauffenburger DA, Wells A (2000) Epidermal growth factor receptor activation of calpain is required for fibroblast motility and occurs via an ERK/MAP kinase signaling pathway. *J Biol Chem* 275:2390-2398.
- Goll DE, Thompson VF, Li H, Wei W, Cong J (2003) The calpain system. *Physiol Rev* 83:731-801.
- Gottlieb RA (2000) Mitochondria: execution central. *FEBS Lett* 482:6-12.
- Graham-Siegenthaler K, Gauthier S, Davies PL, Elce JS (1994) Active recombinant rat calpain II. Bacterially produced large and small subunits associate both in vivo and in vitro. *J Biol Chem* 269:30457-30460.
- Grynspan F, Griffin WB, Mohan PS, Shea TB, Nixon RA (1997a) Calpains and calpastatin in SH-SY5Y neuroblastoma cells during retinoic acid-induced differentiation and neurite outgrowth: comparison with the human brain calpain system. *J Neurosci Res* 48:181-191.
- Grynspan F, Griffin WR, Cataldo A, Katayama S, Nixon RA (1997b) Active site-directed antibodies identify calpain II as an early-appearing and pervasive component of neurofibrillary pathology in Alzheimer's disease. *Brain Res* 763:145-158.
- Guroff G (1964) A Neutral, Calcium-Activated Proteinase from the Soluble Fraction of Rat Brain. *J Biol Chem* 239:149-155.
- Guttmann RP, Elce JS, Bell PD, Isbell JC, Johnson GV (1997) Oxidation inhibits substrate proteolysis by calpain I but not autolysis. *J Biol Chem* 272:2005-2012.
- Hacki J, Egger L, Monney L, Conus S, Rosse T, Fellay I, Borner C (2000) Apoptotic crosstalk between the endoplasmic reticulum and mitochondria controlled by Bcl-2. *Oncogene* 19:2286-2295.
- Haim K, Ben-Aharon I, Shalgi R (2006) Expression and immunolocalization of the calpain-calpastatin system during parthenogenetic activation and fertilization in the rat egg. *Reproduction* 131:35-43.

- Hajnóczky G, Csordas G, Das S, Garcia-Perez C, Saotome M, Sinha Roy S, Yi M (2006) Mitochondrial calcium signalling and cell death: approaches for assessing the role of mitochondrial Ca^{2+} uptake in apoptosis. *Cell Calcium* 40:553-560.
- Halestrap AP (1999) The mitochondrial permeability transition: its molecular mechanism and role in reperfusion injury. *Biochem Soc Symp* 66:181-203.
- Hall ED, Sullivan PG, Gibson TR, Pavel KM, Thompson BM, Scheff SW (2005) Spatial and temporal characteristics of neurodegeneration after controlled cortical impact in mice: more than a focal brain injury. *J Neurotrauma* 22:252-265.
- Hanna RA, Garcia-Diaz BE, Davies PL (2007) Calpastatin simultaneously binds four calpains with different kinetic constants. *FEBS Lett* 581:2894-2898.
- Hanna RA, Campbell RL, Davies PL (2008) Calcium-bound structure of calpain and its mechanism of inhibition by calpastatin. *Nature* 456:409-412.
- Happel RD, Smith KP, Banik NL, Powers JM, Hogan EL, Balentine JD (1981) Ca^{2+} -accumulation in experimental spinal cord trauma. *Brain Res* 211:476-479.
- Harraz MM, Dawson TM, Dawson VL (2008) Advances in neuronal cell death 2007. *Stroke* 39:286-288.
- Harriman JF, Liu XL, Aleo MD, Machaca K, Schnellmann RG (2002) Endoplasmic reticulum Ca^{2+} signaling and calpains mediate renal cell death. *Cell Death Differ* 9:734-741.
- Hasselgren PO (1999) Pathways of muscle protein breakdown in injury and sepsis. *Curr Opin Clin Nutr Metab Care* 2:155-160.
- Haworth RA, Hunter DR (1979) The Ca^{2+} -induced membrane transition in mitochondria. II. Nature of the Ca^{2+} trigger site. *Arch Biochem Biophys* 195:460-467.
- Hegde R, Srinivasula SM, Zhang Z, Wassell R, Mukattash R, Cilenti L, DuBois G, Lazebnik Y, Zervos AS, Fernandes-Alnemri T, Alnemri ES (2002) Identification of Omi/HtrA2 as a mitochondrial apoptotic serine protease that disrupts inhibitor of apoptosis protein-caspase interaction. *J Biol Chem* 277:432-438.
- Herrmann JM, Hell K (2005) Chopped, trapped or tacked--protein translocation into the IMS of mitochondria. *Trends Biochem Sci* 30:205-211.
- Hong SC, Goto Y, Lanzino G, Soleau S, Kassell NF, Lee KS (1994) Neuroprotection with a calpain inhibitor in a model of focal cerebral ischemia. *Stroke* 25:663-669.
- Hood JL, Brooks WH, Roszman TL (2004) Differential compartmentalization of the calpain/calpastatin network with the endoplasmic reticulum and Golgi apparatus. *J Biol Chem* 279:43126-43135.
- Hood JL, Logan BB, Sinai AP, Brooks WH, Roszman TL (2003) Association of the calpain/calpastatin network with subcellular organelles. *Biochem Biophys Res Commun* 310:1200-1212.
- Horikawa Y et al. (2000) Genetic variation in the gene encoding calpain-10 is associated with type 2 diabetes mellitus. *Nat Genet* 26:163-175.
- Hosfield CM, Elce JS, Davies PL, Jia Z (1999) Crystal structure of calpain reveals the structural basis for Ca^{2+} -dependent protease activity and a novel mode of enzyme activation. *EMBO J* 18:6880-6889.
- Hou ST, Jiang SX, Desbois A, Huang D, Kelly J, Tessier L, Karchewski L, Kappler J (2006) Calpain-cleaved collapsin response mediator protein-3 induces neuronal death after glutamate toxicity and cerebral ischemia. *J Neurosci* 26:2241-2249.
- Huang C, Jacobson K, Schaller MD (2004) MAP kinases and cell migration. *J Cell Sci* 117:4619-4628.
- Huang Y, Wang KK (2001) The calpain family and human disease. *Trends Mol Med* 7:355-362.

- Hulbert AJ, Turner N, Hinde J, Else P, Guderley H (2006) How might you compare mitochondria from different tissues and different species? *J Comp Physiol B* 176:93-105.
- Ichas F, Mazat JP (1998) From calcium signaling to cell death: two conformations for the mitochondrial permeability transition pore. Switching from low- to high-conductance state. *Biochim Biophys Acta* 1366:33-50.
- Ichas F, Jouaville LS, Mazat JP (1997) Mitochondria are excitable organelles capable of generating and conveying electrical and calcium signals. *Cell* 89:1145-1153.
- Imajoh S, Suzuki K (1985) Reversible interaction between Ca^{2+} -activated neutral protease (CANP) and its endogenous inhibitor. *FEBS Lett* 187:47-50.
- Imajoh S, Kawasaki H, Suzuki K (1986) The amino-terminal hydrophobic region of the small subunit of calcium-activated neutral protease (CANP) is essential for its activation by phosphatidylinositol. *J Biochem* 99:1281-1284.
- Imajoh S, Kawasaki H, Emori Y, Suzuki K (1987) Calcium-activated neutral protease inhibitor from rabbit erythrocytes lacks the N-terminal region of the liver inhibitor but retains three inhibitory units. *Biochem Biophys Res Commun* 146:630-637.
- Inazawa J, Nakagawa H, Misawa S, Abe T, Minoshima S, Fukuyama R, Maki M, Murachi T, Hatanaka M, Shimizu N (1990) Assignment of the human calpastatin gene (CAST) to chromosome 5 at region q14----q22. *Cytogenet Cell Genet* 54:156-158.
- Ishihara N, Takagi N, Niimura M, Takagi K, Nakano M, Tanonaka K, Funakoshi H, Matsumoto K, Nakamura T, Takeo S (2005) Inhibition of apoptosis-inducing factor translocation is involved in protective effects of hepatocyte growth factor against excitotoxic cell death in cultured hippocampal neurons. *J Neurochem* 95:1277-1286.
- Iwasaki Y (1987) [Experimental studies on acute spinal cord injury]. *Hokkaido Igaku Zasshi* 62:820-829.
- Iwasaki Y, Iizuka H, Yamamoto T, Konno H, Kadoya S (1985) Alleviation of axonal damage in acute spinal cord injury by a protease inhibitor: automated morphometric analysis of drug-effects. *Brain Res* 347:124-126.
- Iwasaki Y, Yamamoto H, Iizuka H, Yamamoto T, Konno H (1987) Suppression of neurofilament degradation by protease inhibitors in experimental spinal cord injury. *Brain Res* 406:99-104.
- Joshi A, Bondada V, Geddes JW (2009) Mitochondrial mu-calpain is not involved in the processing of apoptosis-inducing factor. *Exp Neurol* 218:221-227.
- Joza N, Oudit GY, Brown D, Benit P, Kassiri Z, Vahsen N, Benoit L, Patel MM, Nowikovsky K, Vassault A, Backx PH, Wada T, Kroemer G, Rustin P, Penninger JM (2005) Muscle-specific loss of apoptosis-inducing factor leads to mitochondrial dysfunction, skeletal muscle atrophy, and dilated cardiomyopathy. *Mol Cell Biol* 25:10261-10272.
- Joza N, Susin SA, Daugas E, Stanford WL, Cho SK, Li CY, Sasaki T, Elia AJ, Cheng HY, Ravagnan L, Ferri KF, Zamzami N, Wakeham A, Hakem R, Yoshida H, Kong YY, Mak TW, Zuniga-Pflucker JC, Kroemer G, Penninger JM (2001) Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. *Nature* 410:549-554.
- Jurewicz A, Matysiak M, Tybor K, Kilianek L, Raine CS, Selmaj K (2005) Tumour necrosis factor-induced death of adult human oligodendrocytes is mediated by apoptosis inducing factor. *Brain* 128:2675-2688.
- Kampf A, Posmantur R, Nixon R, Grynspan F, Zhao X, Liu SJ, Newcomb JK, Clifton GL, Hayes RL (1996) mu-calpain activation and calpain-mediated cytoskeletal proteolysis following traumatic brain injury. *J Neurochem* 67:1575-1583.

- Kar P, Chakraborti T, Samanta K, Chakraborti S (2008) Submitochondrial localization of associated mu-calpain and calpastatin. *Arch Biochem Biophys* 470:176-186.
- Kar P, Chakraborti T, Samanta K, Chakraborti S (2009) mu-Calpain mediated cleavage of the Na⁺/Ca²⁺ exchanger in isolated mitochondria under A23187 induced Ca²⁺ stimulation. *Arch Biochem Biophys* 482:66-76.
- Kar P, Chakraborti T, Roy S, Choudhury R, Chakraborti S (2007) Identification of calpastatin and mu-calpain and studies of their association in pulmonary smooth muscle mitochondria. *Arch Biochem Biophys* 466:290-299.
- Kazanis I (2005) CNS injury research; reviewing the last decade: methodological errors and a proposal for a new strategy. *Brain Res Brain Res Rev* 50:377-386.
- Kermer P, Klocker N, Bahr M (1999) Neuronal death after brain injury. Models, mechanisms, and therapeutic strategies in vivo. *Cell Tissue Res* 298:383-395.
- Kerr PM, Suleiman MS, Halestrap AP (1999) Reversal of permeability transition during recovery of hearts from ischemia and its enhancement by pyruvate. *Am J Physiol* 276:H496-502.
- Kim JS, He L, Lemasters JJ (2003) Mitochondrial permeability transition: a common pathway to necrosis and apoptosis. *Biochem Biophys Res Commun* 304:463-470.
- Kindler DD, Thiffault C, Solenski NJ, Dennis J, Kostecki V, Jenkins R, Keeney PM, Bennett JP, Jr. (2003) Neurotoxic nitric oxide rapidly depolarizes and permeabilizes mitochondria by dynamically opening the mitochondrial transition pore. *Mol Cell Neurosci* 23:559-573.
- Kitamura Y, Miyamura A, Takata K, Inden M, Tsuchiya D, Nakamura K, Taniguchi T (2003) Possible involvement of both endoplasmic reticulum-and mitochondria-dependent pathways in thapsigargin-induced apoptosis in human neuroblastoma SH-SY5Y cells. *J Pharmacol Sci* 92:228-236.
- Klein JA, Longo-Guess CM, Rossmann MP, Seburn KL, Hurd RE, Frankel WN, Bronson RT, Ackerman SL (2002) The harlequin mouse mutation downregulates apoptosis-inducing factor. *Nature* 419:367-374.
- Klingenberg M (1980) The ADP-ATP translocation in mitochondria, a membrane potential controlled transport. *J Membr Biol* 56:97-105.
- Kokoszka JE, Waymire KG, Levy SE, Sligh JE, Cai J, Jones DP, MacGregor GR, Wallace DC (2004) The ADP/ATP translocator is not essential for the mitochondrial permeability transition pore. *Nature* 427:461-465.
- Konig N, Raynaud F, Feane H, Durand M, Mestre-Frances N, Rossel M, Ouali A, Benyamin Y (2003) Calpain 3 is expressed in astrocytes of rat and *Microcebus* brain. *J Chem Neuroanat* 25:129-136.
- Konno T, Tanaka N, Kataoka M, Takano E, Maki M (1997) A circular dichroism study of preferential hydration and alcohol effects on a denatured protein, pig calpastatin domain I. *Biochim Biophys Acta* 1342:73-82.
- Kroemer G, Reed JC (2000) Mitochondrial control of cell death. *Nat Med* 6:513-519.
- Kroemer G, Martin SJ (2005) Caspase-independent cell death. *Nat Med* 11:725-730.
- Kroemer G, Galluzzi L, Brenner C (2007) Mitochondrial membrane permeabilization in cell death. *Physiol Rev* 87:99-163.
- Kroemer G et al. (2009) Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. *Cell Death Differ* 16:3-11.
- Ku DH, Kagan J, Chen ST, Chang CD, Baserga R, Wurzel J (1990) The human fibroblast adenine nucleotide translocator gene. Molecular cloning and sequence. *J Biol Chem* 265:16060-16063.
- Kuboki M, Ishii H, Horie S, Kazama M (1992) Procalpain I in cytoplasm is translocated onto plasma and granule membranes during platelet stimulation with thrombin

- and then activated on the membranes. *Biochem Biophys Res Commun* 185:1122-1127.
- Kuchay SM, Kim N, Grunz EA, Fay WP, Chishti AH (2007) Double knockouts reveal that protein tyrosine phosphatase 1B is a physiological target of calpain-1 in platelets. *Mol Cell Biol* 27:6038-6052.
- Kumamoto T, Ueyama H, Sugihara R, Kominami E, Goll DE, Tsuda T (1997) Calpain and cathepsins in the skeletal muscle of inflammatory myopathies. *Eur Neurol* 37:176-181.
- Kumamoto T, Ueyama H, Watanabe S, Yoshioka K, Miike T, Goll DE, Ando M, Tsuda T (1995) Immunohistochemical study of calpain and its endogenous inhibitor in the skeletal muscle of muscular dystrophy. *Acta Neuropathol* 89:399-403.
- Kupina NC, Nath R, Bernath EE, Inoue J, Mitsuyoshi A, Yuen PW, Wang KK, Hall ED (2001) The novel calpain inhibitor SJA6017 improves functional outcome after delayed administration in a mouse model of diffuse brain injury. *J Neurotrauma* 18:1229-1240.
- Landshamer S, Hoehn M, Barth N, Duvezin-Caubet S, Schwake G, Tobaben S, Kazhdan I, Becattini B, Zahler S, Vollmar A, Pellicchia M, Reichert A, Plesnila N, Wagner E, Culmsee C (2008) Bid-induced release of AIF from mitochondria causes immediate neuronal cell death. *Cell Death Differ* 15:1553-1563.
- Lane RD, Allan DM, Mellgren RL (1992) A comparison of the intracellular distribution of mu-calpain, m-calpain, and calpastatin in proliferating human A431 cells. *Exp Cell Res* 203:5-16.
- Laporte C, Kosta A, Klein G, Aubry L, Lam D, Tresse E, Luciani MF, Golstein P (2007) A necrotic cell death model in a protist. *Cell Death Differ* 14:266-274.
- Lee S, Kim S, Sun X, Lee JH, Cho H (2007) Cell cycle-dependent mitochondrial biogenesis and dynamics in mammalian cells. *Biochem Biophys Res Commun* 357:111-117.
- Lee ST, Chu K, Park JE, Kang L, Ko SY, Jung KH, Kim M (2006) Memantine reduces striatal cell death with decreasing calpain level in 3-nitropropionic model of Huntington's disease. *Brain Res* 1118:199-207.
- Lee WJ, Hatanaka M, Maki M (1992a) Multiple forms of rat calpastatin cDNA in the coding region of functionally unknown amino-terminal domain. *Biochim Biophys Acta* 1129:251-253.
- Lee WJ, Ma H, Takano E, Yang HQ, Hatanaka M, Maki M (1992b) Molecular diversity in amino-terminal domains of human calpastatin by exon skipping. *J Biol Chem* 267:8437-8442.
- Lenardo MJ, Baltimore D (1989) NF-kappa B: a pleiotropic mediator of inducible and tissue-specific gene control. *Cell* 58:227-229.
- Li H, Thompson VF, Goll DE (2004) Effects of autolysis on properties of mu- and m-calpain. *Biochim Biophys Acta* 1691:91-103.
- Li LY, Luo X, Wang X (2001) Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature* 412:95-99.
- Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 91:479-489.
- Li W, Srinivasula SM, Chai J, Li P, Wu JW, Zhang Z, Alnemri ES, Shi Y (2002) Structural insights into the pro-apoptotic function of mitochondrial serine protease HtrA2/Omi. *Nat Struct Biol* 9:436-441.
- Lin GD, Chattopadhyay D, Maki M, Wang KK, Carson M, Jin L, Yuen PW, Takano E, Hatanaka M, DeLucas LJ, Narayana SV (1997) Crystal structure of calcium

- bound domain VI of calpain at 1.9 Å resolution and its role in enzyme assembly, regulation, and inhibitor binding. *Nat Struct Biol* 4:539-547.
- Liou AK, Zhou Z, Pei W, Lim TM, Yin XM, Chen J (2005) BimEL up-regulation potentiates AIF translocation and cell death in response to MPTP. *Faseb J* 19:1350-1352.
- Lipton SA, Rosenberg PA (1994) Excitatory amino acids as a final common pathway for neurologic disorders. *N Engl J Med* 330:613-622.
- Lipton SA, Bossy-Wetzel E (2002) Dueling activities of AIF in cell death versus survival: DNA binding and redox activity. *Cell* 111:147-150.
- Liu D, Thangnipon W, McAdoo DJ (1991) Excitatory amino acids rise to toxic levels upon impact injury to the rat spinal cord. *Brain Res* 547:344-348.
- Liu X, Van Vleet T, Schnellmann RG (2004) The role of calpain in oncotic cell death. *Annu Rev Pharmacol Toxicol* 44:349-370.
- Liu X, Rainey JJ, Harriman JF, Schnellmann RG (2001) Calpains mediate acute renal cell death: role of autolysis and translocation. *Am J Physiol Renal Physiol* 281:F728-738.
- Liu X, Kim CN, Yang J, Jemmerson R, Wang X (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* 86:147-157.
- Loeffler M, Daugas E, Susin SA, Zamzami N, Metivier D, Nieminen AL, Brothers G, Penninger JM, Kroemer G (2001) Dominant cell death induction by extramitochondrially targeted apoptosis-inducing factor. *Faseb J* 15:758-767.
- Lynch G, Baudry M (1987) Brain spectrin, calpain and long-term changes in synaptic efficacy. *Brain Res Bull* 18:809-815.
- Ma H, Fukiage C, Kim YH, Duncan MK, Reed NA, Shih M, Azuma M, Shearer TR (2001) Characterization and expression of calpain 10. A novel ubiquitous calpain with nuclear localization. *J Biol Chem* 276:28525-28531.
- Mader JS, Mookherjee N, Hancock RE, Bleackley RC (2009) The human host defense peptide LL-37 induces apoptosis in a calpain- and apoptosis-inducing factor-dependent manner involving Bax activity. *Mol Cancer Res* 7:689-702.
- Maki M, Narayana SV, Hitomi K (1997) A growing family of the Ca²⁺-binding proteins with five EF-hand motifs. *Biochem J* 328 (Pt 2):718-720.
- Maki M, Takano E, Mori H, Sato A, Murachi T, Hatanaka M (1987) All four internally repetitive domains of pig calpastatin possess inhibitory activities against calpains I and II. *FEBS Lett* 223:174-180.
- Maki M, Ma H, Takano E, Adachi Y, Lee WJ, Hatanaka M, Murachi T (1991) Calpastatins: biochemical and molecular biological studies. *Biomed Biochim Acta* 50:509-516.
- Mannella CA, Marko M, Buttle K (1997) Reconsidering mitochondrial structure: new views of an old organelle. *Trends Biochem Sci* 22:37-38.
- Mannella CA, Buttle K, Rath BK, Marko M (1998) Electron microscopic tomography of rat-liver mitochondria and their interaction with the endoplasmic reticulum. *Biofactors* 8:225-228.
- Mannella CA, Marko M, Penczek P, Barnard D, Frank J (1994) The internal compartmentation of rat-liver mitochondria: tomographic study using the high-voltage transmission electron microscope. *Microsc Res Tech* 27:278-283.
- Mark RJ, Pang Z, Geddes JW, Uchida K, Mattson MP (1997) Amyloid beta-peptide impairs glucose transport in hippocampal and cortical neurons: involvement of membrane lipid peroxidation. *J Neurosci* 17:1046-1054.
- Martin W, Koonin EV (2006) A positive definition of prokaryotes. *Nature* 442:868.
- Mathur P, Gupta SK, Wegener AR, Breipohl W, Ahrend MH, Sharma YD, Gupta YK, Vajpayee RB (2000) Comparison of various calpain inhibitors in reduction of light

- scattering, protein precipitation and nuclear cataract in vitro. *Curr Eye Res* 21:926-933.
- Matsumori Y, Hong SM, Aoyama K, Fan Y, Kayama T, Sheldon RA, Vexler ZS, Ferriero DM, Weinstein PR, Liu J (2005) Hsp70 overexpression sequesters AIF and reduces neonatal hypoxic/ischemic brain injury. *J Cereb Blood Flow Metab* 25:899-910.
- McAdoo DJ, Xu GY, Robak G, Hughes MG (1999) Changes in amino acid concentrations over time and space around an impact injury and their diffusion through the rat spinal cord. *Exp Neurol* 159:538-544.
- Mehdi S, Angelastro MR, Wiseman JS, Bey P (1988) Inhibition of the proteolysis of rat erythrocyte membrane proteins by a synthetic inhibitor of calpain. *Biochem Biophys Res Commun* 157:1117-1123.
- Meli E, Pangallo M, Picca R, Baronti R, Moroni F, Pellegrini-Giampietro DE (2004) Differential role of poly(ADP-ribose) polymerase-1 in apoptotic and necrotic neuronal death induced by mild or intense NMDA exposure in vitro. *Mol Cell Neurosci* 25:172-180.
- Mellgren RL (1988) On the mechanism of binding of calpastatin, the protein inhibitor of calpains, to biologic membranes. *Biochem Biophys Res Commun* 150:170-176.
- Mellgren RL (1991) Proteolysis of nuclear proteins by mu-calpain and m-calpain. *J Biol Chem* 266:13920-13924.
- Mellgren RL, Lane RD, Kakar SS (1987) A sarcolemma-associated inhibitor is capable of modulating calcium-dependent proteinase activity. *Biochim Biophys Acta* 930:370-377.
- Mellgren RL, Renno WM, Lane RD (1989) The non-lysosomal, calcium-dependent proteolytic system of mammalian cells. *Revis Biol Celular* 20:139-159.
- Melloni E, Michetti M, Salamino F, Minafra R, Pontremoli S (1996) Modulation of the calpain autoproteolysis by calpastatin and phospholipids. *Biochem Biophys Res Commun* 229:193-197.
- Melloni E, Michetti M, Salamino F, Sparatore B, Pontremoli S (1998a) Mechanism of action of a new component of the Ca(2+)-dependent proteolytic system in rat brain: the calpain activator. *Biochem Biophys Res Commun* 249:583-588.
- Melloni E, Aversa M, Salamino F, Sparatore B, Minafra R, Pontremoli S (2000) Acyl-CoA-binding protein is a potent m-calpain activator. *J Biol Chem* 275:82-86.
- Melloni E, De Tullio R, Aversa M, Tedesco I, Salamino F, Sparatore B, Pontremoli S (1998b) Properties of calpastatin forms in rat brain. *FEBS Lett* 431:55-58.
- Michetti M, Salamino F, Minafra R, Melloni E, Pontremoli S (1997) Calcium-binding properties of human erythrocyte calpain. *Biochem J* 325 (Pt 3):721-726.
- Miyoshi H, Umeshita K, Sakon M, Imajoh-Ohmi S, Fujitani K, Gotoh M, Oiki E, Kambayashi J, Monden M (1996) Calpain activation in plasma membrane bleb formation during tert-butyl hydroperoxide-induced rat hepatocyte injury. *Gastroenterology* 110:1897-1904.
- Modjtahedi N, Giordanetto F, Madeo F, Kroemer G (2006) Apoptosis-inducing factor: vital and lethal. *Trends Cell Biol* 16:264-272.
- Moldoveanu T, Gehring K, Green DR (2008) Concerted multi-pronged attack by calpastatin to occlude the catalytic cleft of heterodimeric calpains. *Nature* 456:404-408.
- Molinari M, Carafoli E (1997) Calpain: a cytosolic proteinase active at the membranes. *J Membr Biol* 156:1-8.
- Molinari M, Anagli J, Carafoli E (1994) Ca(2+)-activated neutral protease is active in the erythrocyte membrane in its nonautolyzed 80-kDa form. *J Biol Chem* 269:27992-27995.

- Moriya T, Hassan AZ, Young W, Chesler M (1994) Dynamics of extracellular calcium activity following contusion of the rat spinal cord. *J Neurotrauma* 11:255-263.
- Moubarak RS, Yuste VJ, Artus C, Bouharrou A, Greer PA, Menissier-de Murcia J, Susin SA (2007) Sequential Activation of PARP-1, Calpains, and Bax is Essential in AIF-Mediated Programmed Necrosis. *Mol Cell Biol*.
- Mundo E, Soldati L, Bellodi L, Bianchi G (1997) The calpain-calpastatin system in obsessive-compulsive disorder. *Biol Psychiatry* 42:228-229.
- Muntener K, Zwicky R, Csucs G, Rohrer J, Baici A (2004) Exon skipping of cathepsin B: mitochondrial targeting of a lysosomal peptidase provokes cell death. *J Biol Chem* 279:41012-41017.
- Murachi T, Takano E, Maki M, Adachi Y, Hatanaka M (1989) Cloning and expression of the genes for calpains and calpastatins. *Biochem Soc Symp* 55:29-44.
- Murakami T, Hatanaka M, Murachi T (1981) The cytosol of human erythrocytes contains a highly Ca^{2+} -sensitive thiol protease (calpain I) and its specific inhibitor protein (calpastatin). *J Biochem* 90:1809-1816.
- Muruganandan S, Cribb AE (2006) Calpain-induced endoplasmic reticulum stress and cell death following cytotoxic damage to renal cells. *Toxicol Sci* 94:118-128.
- Nakagawa T, Yuan J (2000) Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. *J Cell Biol* 150:887-894.
- Nakagawa T, Shimizu S, Watanabe T, Yamaguchi O, Otsu K, Yamagata H, Inohara H, Kubo T, Tsujimoto Y (2005) Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. *Nature* 434:652-658.
- Nakamura Y, Fukiage C, Shih M, Ma H, David LL, Azuma M, Shearer TR (2000) Contribution of calpain Lp82-induced proteolysis to experimental cataractogenesis in mice. *Invest Ophthalmol Vis Sci* 41:1460-1466.
- Nath R, Raser KJ, Hajimohammadreza I, Wang KK (1997) Thapsigargin induces apoptosis in SH-SY5Y neuroblastoma cells and cerebrocortical cultures. *Biochem Mol Biol Int* 43:197-205.
- Neupert W, Herrmann JM (2007) Translocation of proteins into mitochondria. *Annu Rev Biochem* 76:723-749.
- Niapour M, Yu Y, Berger SA (2008) Regulation of calpain activity by c-Myc through calpastatin and promotion of transformation in c-Myc-negative cells by calpastatin suppression. *J Biol Chem* 283:21371-21381.
- Nicolli A, Petronilli V, Bernardi P (1993) Modulation of the mitochondrial cyclosporin A-sensitive permeability transition pore by matrix pH. Evidence that the pore open-closed probability is regulated by reversible histidine protonation. *Biochemistry* 32:4461-4465.
- Nixon RA (1986) Fodrin degradation by calcium-activated neutral proteinase (CANP) in retinal ganglion cell neurons and optic glia: preferential localization of CANP activities in neurons. *J Neurosci* 6:1264-1271.
- Norberg E, Gogvadze V, Ott M, Horn M, Uhlen P, Orrenius S, Zhivotovsky B (2008) An increase in intracellular Ca^{2+} is required for the activation of mitochondrial calpain to release AIF during cell death. *Cell Death Differ*.
- Ohno S, Emori Y, Suzuki K (1986) Nucleotide sequence of a cDNA coding for the small subunit of human calcium-dependent protease. *Nucleic Acids Res* 14:5559.
- Okitani (1976) Intracellular inhibitor of a calcium-activated protease involved in myofibrillar protein turnover. In: *Federation Proc*, p 1746.
- Olney JW (1969) Brain lesions, obesity, and other disturbances in mice treated with monosodium glutamate. *Science* 164:719-721.

- Ono Y, Sorimachi H, Suzuki K (1999) New aspect of the research on limb-girdle muscular dystrophy 2A: a molecular biologic and biochemical approach to pathology. *Trends Cardiovasc Med* 9:114-118.
- Otera H, Ohsakaya S, Nagaura Z, Ishihara N, Mihara K (2005) Export of mitochondrial AIF in response to proapoptotic stimuli depends on processing at the intermembrane space. *Embo J* 24:1375-1386.
- Otsuka Y, Goll DE (1987) Purification of the Ca²⁺-dependent proteinase inhibitor from bovine cardiac muscle and its interaction with the millimolar Ca²⁺-dependent proteinase. *J Biol Chem* 262:5839-5851.
- Ozaki T, Yamashita T, Ishiguro SI (2008) ERp57-associated mitochondrial mu-calpain truncates apoptosis-inducing factor. *Biochim Biophys Acta*.
- Ozaki T, Tomita H, Tamai M, Ishiguro S (2007) Characteristics of mitochondrial calpains. *J Biochem* 142:365-376.
- Palade GE (1952) The fine structure of mitochondria. *Anat Rec* 114:427-451.
- Pang Z, Geddes JW (1997) Mechanisms of cell death induced by the mitochondrial toxin 3-nitropropionic acid: acute excitotoxic necrosis and delayed apoptosis. *J Neurosci* 17:3064-3073.
- Pang Z, Bondada V, Sengoku T, Siman R, Geddes JW (2003) Calpain facilitates the neuron death induced by 3-nitropropionic acid and contributes to the necrotic morphology. *J Neuropathol Exp Neurol* 62:633-643.
- Panov A, Dikalov S, Shalbuyeva N, Hemendinger R, Greenamyre JT, Rosenfeld J (2007) Species- and tissue-specific relationships between mitochondrial permeability transition and generation of ROS in brain and liver mitochondria of rats and mice. *Am J Physiol Cell Physiol* 292:C708-718.
- Park E, Velumian AA, Fehlings MG (2004) The role of excitotoxicity in secondary mechanisms of spinal cord injury: a review with an emphasis on the implications for white matter degeneration. *J Neurotrauma* 21:754-774.
- Patel YM, Lane MD (2000) Mitotic clonal expansion during preadipocyte differentiation: calpain-mediated turnover of p27. *J Biol Chem* 275:17653-17660.
- Penninger JM, Kroemer G (2003) Mitochondria, AIF and caspases--rivaling for cell death execution. *Nat Cell Biol* 5:97-99.
- Perkins GA, Frey TG (2000) Recent structural insight into mitochondria gained by microscopy. *Micron* 31:97-111.
- Petit PX, Goubern M, Diolez P, Susin SA, Zamzami N, Kroemer G (1998) Disruption of the outer mitochondrial membrane as a result of large amplitude swelling: the impact of irreversible permeability transition. *FEBS Lett* 426:111-116.
- Petronilli V, Costantini P, Scorrano L, Colonna R, Passamonti S, Bernardi P (1994) The voltage sensor of the mitochondrial permeability transition pore is tuned by the oxidation-reduction state of vicinal thiols. Increase of the gating potential by oxidants and its reversal by reducing agents. *J Biol Chem* 269:16638-16642.
- Pettus EH, Christman CW, Giebel ML, Povlishock JT (1994) Traumatically induced altered membrane permeability: its relationship to traumatically induced reactive axonal change. *J Neurotrauma* 11:507-522.
- Pike BR, Zhao X, Newcomb JK, Posmantur RM, Wang KK, Hayes RL (1998) Regional calpain and caspase-3 proteolysis of alpha-spectrin after traumatic brain injury. *Neuroreport* 9:2437-2442.
- Pike BR, Flint J, Dutta S, Johnson E, Wang KK, Hayes RL (2001) Accumulation of non-erythroid alpha II-spectrin and calpain-cleaved alpha II-spectrin breakdown products in cerebrospinal fluid after traumatic brain injury in rats. *J Neurochem* 78:1297-1306.

- Pizzo P, Pozzan T (2007) Mitochondria-endoplasmic reticulum choreography: structure and signaling dynamics. *Trends Cell Biol* 17:511-517.
- Plesnila N, Zhu C, Culmsee C, Groger M, Moskowitz MA, Blomgren K (2004) Nuclear translocation of apoptosis-inducing factor after focal cerebral ischemia. *J Cereb Blood Flow Metab* 24:458-466.
- Polster BM, Basanez G, Etxebarria A, Hardwick JM, Nicholls DG (2005) Calpain I induces cleavage and release of apoptosis-inducing factor from isolated mitochondria. *J Biol Chem* 280:6447-6454.
- Pontremoli S, Salamino F, Sparatore B, De Tullio R, Pontremoli R, Melloni E (1988) Characterization of the calpastatin defect in erythrocytes from patients with essential hypertension. *Biochem Biophys Res Commun* 157:867-874.
- Posmantur R, Kampfl A, Siman R, Liu J, Zhao X, Clifton GL, Hayes RL (1997) A calpain inhibitor attenuates cortical cytoskeletal protein loss after experimental traumatic brain injury in the rat. *Neuroscience* 77:875-888.
- Potter DA, Tirnauer JS, Janssen R, Croall DE, Hughes CN, Fiacco KA, Mier JW, Maki M, Herman IM (1998) Calpain regulates actin remodeling during cell spreading. *J Cell Biol* 141:647-662.
- Poyton RO, McEwen JE (1996) Crosstalk between nuclear and mitochondrial genomes. *Annu Rev Biochem* 65:563-607.
- Prehn JH, Bindokas VP, Marcuccilli CJ, Krajewski S, Reed JC, Miller RJ (1994) Regulation of neuronal Bcl2 protein expression and calcium homeostasis by transforming growth factor type beta confers wide-ranging protection on rat hippocampal neurons. *Proc Natl Acad Sci U S A* 91:12599-12603.
- Rami A, Agarwal R, Botez G, Winckler J (2000) mu-Calpain activation, DNA fragmentation, and synergistic effects of caspase and calpain inhibitors in protecting hippocampal neurons from ischemic damage. *Brain Res* 866:299-312.
- Rasmussen N (1995) Mitochondrial structure and the practice of cell biology in the 1950s. *J Hist Biol* 28:381-429.
- Ray SK, Hogan EL, Banik NL (2003) Calpain in the pathophysiology of spinal cord injury: neuroprotection with calpain inhibitors. *Brain Res Brain Res Rev* 42:169-185.
- Ray SK, Matzelle DC, Wilford GG, Hogan EL, Banik NL (2000) E-64-d prevents both calpain upregulation and apoptosis in the lesion and penumbra following spinal cord injury in rats. *Brain Res* 867:80-89.
- Ray SK, Matzelle DD, Wilford GG, Hogan EL, Banik NL (2001a) Cell death in spinal cord injury (SCI) requires de novo protein synthesis. Calpain inhibitor E-64-d provides neuroprotection in SCI lesion and penumbra. *Ann N Y Acad Sci* 939:436-449.
- Ray SK, Matzelle DD, Wilford GG, Hogan EL, Banik NL (2001b) Inhibition of calpain-mediated apoptosis by E-64 d-reduced immediate early gene (IEG) expression and reactive astrogliosis in the lesion and penumbra following spinal cord injury in rats. *Brain Res* 916:115-126.
- Ray SK, Shields DC, Saido TC, Matzelle DC, Wilford GG, Hogan EL, Banik NL (1999) Calpain activity and translational expression increased in spinal cord injury. *Brain Res* 816:375-380.
- Rego AC, Oliveira CR (2003) Mitochondrial dysfunction and reactive oxygen species in excitotoxicity and apoptosis: implications for the pathogenesis of neurodegenerative diseases. *Neurochem Res* 28:1563-1574.
- Reichert AS, Neupert W (2002) Contact sites between the outer and inner membrane of mitochondria-role in protein transport. *Biochim Biophys Acta* 1592:41-49.
- Richard I, Broux O, Allamand V, Fougereousse F, Chiannikulchai N, Bourg N, Brenguier L, Devaud C, Pasturaud P, Roudaut C, et al. (1995) Mutations in the proteolytic enzyme calpain 3 cause limb-girdle muscular dystrophy type 2A. *Cell* 81:27-40.

- Ritov VB, Tverdislova IL, Avakyan T, Menshikova EV, Leikin Yu N, Bratkovskaya LB, Shimon RG (1992) Alamethicin-induced pore formation in biological membranes. *Gen Physiol Biophys* 11:49-58.
- Roberts-Lewis JM, Savage MJ, Marcy VR, Pinsker LR, Siman R (1994) Immunolocalization of calpain I-mediated spectrin degradation to vulnerable neurons in the ischemic gerbil brain. *J Neurosci* 14:3934-3944.
- Roucou X, Martinou JC (2001) Conformational change of Bax: a question of life or death. *Cell Death Differ* 8:875-877.
- Saatman KE, Graham DI, McIntosh TK (1998) The neuronal cytoskeleton is at risk after mild and moderate brain injury. *J Neurotrauma* 15:1047-1058.
- Saatman KE, Zhang C, Bartus RT, McIntosh TK (2000) Behavioral efficacy of posttraumatic calpain inhibition is not accompanied by reduced spectrin proteolysis, cortical lesion, or apoptosis. *J Cereb Blood Flow Metab* 20:66-73.
- Saatman KE, Bozyczko-Coyne D, Marcy V, Siman R, McIntosh TK (1996a) Prolonged calpain-mediated spectrin breakdown occurs regionally following experimental brain injury in the rat. *J Neuropathol Exp Neurol* 55:850-860.
- Saatman KE, Murai H, Bartus RT, Smith DH, Hayward NJ, Perri BR, McIntosh TK (1996b) Calpain inhibitor AK295 attenuates motor and cognitive deficits following experimental brain injury in the rat. *Proc Natl Acad Sci U S A* 93:3428-3433.
- Saido TC, Sorimachi H, Suzuki K (1994) Calpain: new perspectives in molecular diversity and physiological-pathological involvement. *Faseb J* 8:814-822.
- Saido TC, Suzuki H, Yamazaki H, Tanoue K, Suzuki K (1993a) In situ capture of mu-calpain activation in platelets. *J Biol Chem* 268:7422-7426.
- Saido TC, Yokota M, Nagao S, Yamaura I, Tani E, Tsuchiya T, Suzuki K, Kawashima S (1993b) Spatial resolution of fodrin proteolysis in postischemic brain. *J Biol Chem* 268:25239-25243.
- Saito K, Elce JS, Hamos JE, Nixon RA (1993) Widespread activation of calcium-activated neutral proteinase (calpain) in the brain in Alzheimer disease: a potential molecular basis for neuronal degeneration. *Proc Natl Acad Sci U S A* 90:2628-2632.
- Saito M, Hagiwara T, Aoyagi T, Nagai Y (1972) Leupeptin, a protease inhibitor, inhibits the PHA-stimulated DNA synthesis in guinea pig blood lymphocytes. *Jpn J Exp Med* 42:509-511.
- Salamino F, De Tullio R, Michetti M, Mengotti P, Melloni E, Pontremoli S (1994) Modulation of calpastatin specificity in rat tissues by reversible phosphorylation and dephosphorylation. *Biochem Biophys Res Commun* 199:1326-1332.
- Salamino F, Averna M, Tedesco I, De Tullio R, Melloni E, Pontremoli S (1997) Modulation of rat brain calpastatin efficiency by post-translational modifications. *FEBS Lett* 412:433-438.
- Sanges D, Marigo V (2006) Cross-talk between two apoptotic pathways activated by endoplasmic reticulum stress: differential contribution of caspase-12 and AIF. *Apoptosis* 11:1629-1641.
- Santella L, Kyojuka K, Hoving S, Munchbach M, Quadroni M, Dainese P, Zamparelli C, James P, Carafoli E (2000) Breakdown of cytoskeletal proteins during meiosis of starfish oocytes and proteolysis induced by calpain. *Exp Cell Res* 259:117-126.
- Sasaki T, Kishi M, Saito M, Tanaka T, Higuchi N, Kominami E, Katunuma N, Murachi T (1990) Inhibitory effect of di- and tripeptidyl aldehydes on calpains and cathepsins. *J Enzyme Inhib* 3:195-201.
- Satish L, Blair HC, Glading A, Wells A (2005) Interferon-inducible protein 9 (CXCL11)-induced cell motility in keratinocytes requires calcium flux-dependent activation of mu-calpain. *Mol Cell Biol* 25:1922-1941.

- Sattler R, Tymianski M (2001) Molecular mechanisms of glutamate receptor-mediated excitotoxic neuronal cell death. *Mol Neurobiol* 24:107-129.
- Scarpulla RC (2002) Nuclear activators and coactivators in mammalian mitochondrial biogenesis. *Biochim Biophys Acta* 1576:1-14.
- Schollmeyer JE (1988) Calpain II involvement in mitosis. *Science* 240:911-913.
- Schon EA (2000) Mitochondrial genetics and disease. *Trends Biochem Sci* 25:555-560.
- Schumacher PA, Eubanks JH, Fehlings MG (1999) Increased calpain I-mediated proteolysis, and preferential loss of dephosphorylated NF200, following traumatic spinal cord injury. *Neuroscience* 91:733-744.
- Schumacher PA, Siman RG, Fehlings MG (2000) Pretreatment with calpain inhibitor CEP-4143 inhibits calpain I activation and cytoskeletal degradation, improves neurological function, and enhances axonal survival after traumatic spinal cord injury. *J Neurochem* 74:1646-1655.
- SCI FSC (1998) CDC spinal cord injury fact sheet
- Scorrano L, Ashiya M, Buttle K, Weiler S, Oakes SA, Mannella CA, Korsmeyer SJ (2002) A distinct pathway remodels mitochondrial cristae and mobilizes cytochrome c during apoptosis. *Dev Cell* 2:55-67.
- Sengoku T, Bondada V, Hassane D, Dubal S, Geddes JW (2004) Tat-calpastatin fusion proteins transduce primary rat cortical neurons but do not inhibit cellular calpain activity. *Exp Neurol* 188:161-170.
- Shalbuyeva N, Brustovetsky T, Bolshakov A, Brustovetsky N (2006) Calcium-dependent spontaneously reversible remodeling of brain mitochondria. *J Biol Chem* 281:37547-37558.
- Shea TB, Cressman CM, Spencer MJ, Beermann ML, Nixon RA (1995) Enhancement of neurite outgrowth following calpain inhibition is mediated by protein kinase C. *J Neurochem* 65:517-527.
- Shields DC, Schaecher KE, Saido TC, Banik NL (1999) A putative mechanism of demyelination in multiple sclerosis by a proteolytic enzyme, calpain. *Proc Natl Acad Sci U S A* 96:11486-11491.
- Shulga N, Pastorino JG (2006) Acyl coenzyme A-binding protein augments bid-induced mitochondrial damage and cell death by activating mu-calpain. *J Biol Chem* 281:30824-30833.
- Siman R, Baudry M, Lynch G (1984) Brain fodrin: substrate for calpain I, an endogenous calcium-activated protease. *Proc Natl Acad Sci U S A* 81:3572-3576.
- Siman R, Gall C, Perlmutter LS, Christian C, Baudry M, Lynch G (1985) Distribution of calpain I, an enzyme associated with degenerative activity, in rat brain. *Brain Res* 347:399-403.
- Sims NR (1990) Rapid isolation of metabolically active mitochondria from rat brain and subregions using Percoll density gradient centrifugation. *J Neurochem* 55:698-707.
- Sjostrand FS (1953) Electron microscopy of mitochondria and cytoplasmic double membranes. *Nature* 171:30-32.
- Smith DJ, Ng H, Kluck RM, Nagley P (2008) The mitochondrial gateway to cell death. *IUBMB Life* 60:383-389.
- Sorimachi H, Suzuki K (2001) The structure of calpain. *J Biochem* 129:653-664.
- Sorimachi H, Saido TC, Suzuki K (1994) New Era of Calpain Research - Discovery of Tissue-Specific Calpains. *Febs Letters* 343:1-5.
- Sorimachi H, Ishiura S, Suzuki K (1997a) Structure and physiological function of calpains. *Biochem J* 328 (Pt 3):721-732.

- Sorimachi Y, Harada K, Saido TC, Ono T, Kawashima S, Yoshida K (1997b) Downregulation of calpastatin in rat heart after brief ischemia and reperfusion. *J Biochem (Tokyo)* 122:743-748.
- Springer JE, Azbill RD, Kennedy SE, George J, Geddes JW (1997) Rapid calpain I activation and cytoskeletal protein degradation following traumatic spinal cord injury: attenuation with riluzole pretreatment. *J Neurochem* 69:1592-1600.
- Stoka V, Turk V, Bredesen DE (2006) Differential regulation of the intrinsic pathway of apoptosis in brain and liver during ageing. *FEBS Lett* 580:3739-3745.
- Storer AC, Menard R (1994) Catalytic mechanism in papain family of cysteine peptidases. *Methods Enzymol* 244:486-500.
- Sullivan PG, Rabchevsky AG, Waldmeier PC, Springer JE (2005) Mitochondrial permeability transition in CNS trauma: cause or effect of neuronal cell death? *J Neurosci Res* 79:231-239.
- Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion J, Jacotot E, Costantini P, Loeffler M, Larochette N, Goodlett DR, Aebersold R, Siderovski DP, Penninger JM, Kroemer G (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* 397:441-446.
- Suzuki K (1990) The structure of the calpains and the calpain gene. In: *Intracellular calcium-dependent proteolysis* (Murachi RL MaT, ed), pp 25-35: Baco Raton, FL.
- Suzuki K (1991) Nomenclature of calcium dependent proteinase. *Biomed Biochim Acta* 50:483-484.
- Suzuki K, Sorimachi H (1998) A novel aspect of calpain activation. *FEBS Lett* 433:1-4.
- Suzuki K, Hata S, Kawabata Y, Sorimachi H (2004) Structure, activation, and biology of calpain. *Diabetes* 53 Suppl 1:S12-18.
- Suzuki K, Sorimachi H, Yoshizawa T, Kinbara K, Ishiura S (1995) Calpain - Novel Family Members, Activation, and Physiological-Function. *Biological Chemistry Hoppe-Seyler* 376:523-529.
- Suzuki K, Imajoh S, Emori Y, Kawasaki H, Minami Y, Ohno S (1987) Calcium-activated neutral protease and its endogenous inhibitor. Activation at the cell membrane and biological function. *FEBS Lett* 220:271-277.
- Syntichaki P, Xu K, Driscoll M, Tavernarakis N (2002) Specific aspartyl and calpain proteases are required for neurodegeneration in *C. elegans*. *Nature* 419:939-944.
- Szabadkai G, Bianchi K, Varnai P, De Stefani D, Wieckowski MR, Cavagna D, Nagy AI, Balla T, Rizzuto R (2006) Chaperone-mediated coupling of endoplasmic reticulum and mitochondrial Ca²⁺ channels. *J Cell Biol* 175:901-911.
- Szabo I, Zoratti M (1992) The mitochondrial megachannel is the permeability transition pore. *J Bioenerg Biomembr* 24:111-117.
- Tait SW, Green DR (2008) Caspase-independent cell death: leaving the set without the final cut. *Oncogene* 27:6452-6461.
- Takano E, Murachi T (1982a) Purification and some properties of human erythrocyte calpastatin. *J Biochem* 92:2021-2028.
- Takano E, Murachi T (1982b) Purification and some properties of human erythrocyte calpastatin. *J Biochem (Tokyo)* 92:2021-2028.
- Takano J, Watanabe M, Hitomi K, Maki M (2000) Four types of calpastatin isoforms with distinct amino-terminal sequences are specified by alternative first exons and differentially expressed in mouse tissues. *J Biochem* 128:83-92.
- Takano J, Kawamura T, Murase M, Hitomi K, Maki M (1999) Structure of mouse calpastatin isoforms: implications of species-common and species-specific alternative splicing. *Biochem Biophys Res Commun* 260:339-345.

- Takano J, Tomioka M, Tsubuki S, Higuchi M, Iwata N, Itohara S, Maki M, Saido TC (2005) Calpain mediates excitotoxic DNA fragmentation via mitochondrial pathways in adult brains: evidence from calpastatin mutant mice. *J Biol Chem* 280:16175-16184.
- Takeuchi KH, Saito KI, Nixon RA (1992) Immunoassay and activity of calcium-activated neutral proteinase (mCANP): distribution in soluble and membrane-associated fractions in human and mouse brain. *J Neurochem* 58:1526-1532.
- Tamura T, Akutsu T (2007) Subcellular location prediction of proteins using support vector machines with alignment of block sequences utilizing amino acid composition. *BMC Bioinformatics* 8:466.
- Tator CH, Fehlings MG (1991) Review of the secondary injury theory of acute spinal cord trauma with emphasis on vascular mechanisms. *J Neurosurg* 75:15-26.
- Tavares A, Duque-Magalhaes MC (1991) Demonstration of three calpains in the matrix of rat liver mitochondria. *Biomed Biochim Acta* 50:523-529.
- Taylor RG, Christiansen JA, Goll DE (1991) Immunolocalization of the calpains and calpastatin in human and bovine platelets. *Biomed Biochim Acta* 50:491-498.
- TBI FsC (2000) Data of year 2000 from CDC TBI fact sheet. In.
- Thastrup O, Cullen PJ, Drobak BK, Hanley MR, Dawson AP (1990) Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+} -ATPase. *Proc Natl Acad Sci U S A* 87:2466-2470.
- Todd B, Moore D, Deivanayagam CC, Lin GD, Chattopadhyay D, Maki M, Wang KK, Narayana SV (2003) A structural model for the inhibition of calpain by calpastatin: crystal structures of the native domain VI of calpain and its complexes with calpastatin peptide and a small molecule inhibitor. *J Mol Biol* 328:131-146.
- Tomimatsu Y, Idemoto S, Moriguchi S, Watanabe S, Nakanishi H (2002) Proteases involved in long-term potentiation. *Life Sci* 72:355-361.
- Tompa P, Buzder-Lantos P, Tantos A, Farkas A, Szilagyi A, Banoczy Z, Hudecz F, Friedrich P (2004) On the sequential determinants of calpain cleavage. *J Biol Chem* 279:20775-20785.
- Tsuji T, Shimohama S, Kimura J, Shimizu K (1998) m-Calpain (calcium-activated neutral proteinase) in Alzheimer's disease brains. *Neurosci Lett* 248:109-112.
- Tullio RD, Passalacqua M, Aversa M, Salamino F, Melloni E, Pontremoli S (1999) Changes in intracellular localization of calpastatin during calpain activation. *Biochem J* 343 Pt 2:467-472.
- Uemori T, Shimojo T, Asada K, Asano T, Kimizuka F, Kato I, Maki M, Hatanaka M, Murachi T, Hanzawa H, et al. (1990) Characterization of a functional domain of human calpastatin. *Biochem Biophys Res Commun* 166:1485-1493.
- Vahsen N, Cande C, Briere JJ, Benit P, Joza N, Larochette N, Mastroberardino PG, Pequignot MO, Casares N, Lazar V, Feraud O, Debili N, Wissing S, Engelhardt S, Madeo F, Piacentini M, Penninger JM, Schagger H, Rustin P, Kroemer G (2004) AIF deficiency compromises oxidative phosphorylation. *EMBO J* 23:4679-4689.
- van Gurp M, Festjens N, van Loo G, Saelens X, Vandenabeele P (2003) Mitochondrial intermembrane proteins in cell death. *Biochem Biophys Res Commun* 304:487-497.
- van Loo G, Saelens X, Matthijssens F, Schotte P, Beyaert R, Declercq W, Vandenabeele P (2002a) Caspases are not localized in mitochondria during life or death. *Cell Death Differ* 9:1207-1211.
- Van Loo G, Demol H, van Gurp M, Hoorelbeke B, Schotte P, Beyaert R, Zhivotovsky B, Gevaert K, Declercq W, Vandekerckhove J, Vandenabeele P (2002b) A matrix-

- assisted laser desorption ionization post-source decay (MALDI-PSD) analysis of proteins released from isolated liver mitochondria treated with recombinant truncated Bid. *Cell Death Differ* 9:301-308.
- Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, Moritz RL, Simpson RJ, Vaux DL (2000) Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* 102:43-53.
- Verhagen AM, Silke J, Ekert PG, Pakusch M, Kaufmann H, Connolly LM, Day CL, Tikoo A, Burke R, Wrobel C, Moritz RL, Simpson RJ, Vaux DL (2002) HtrA2 promotes cell death through its serine protease activity and its ability to antagonize inhibitor of apoptosis proteins. *J Biol Chem* 277:445-454.
- Verkhratsky A (2005) Physiology and pathophysiology of the calcium store in the endoplasmic reticulum of neurons. *Physiol Rev* 85:201-279.
- Vieira HL, Haouzi D, El Hamel C, Jacotot E, Belzacq AS, Brenner C, Kroemer G (2000) Permeabilization of the mitochondrial inner membrane during apoptosis: impact of the adenine nucleotide translocator. *Cell Death Differ* 7:1146-1154.
- Vinade L, Petersen JD, Do K, Dosemeci A, Reese TS (2001) Activation of calpain may alter the postsynaptic density structure and modulate anchoring of NMDA receptors. *Synapse* 40:302-309.
- von Heijne G (1986) Mitochondrial targeting sequences may form amphiphilic helices. *EMBO J* 5:1335-1342.
- Waghray A, Wang DS, McKinsey D, Hayes RL, Wang KK (2004) Molecular cloning and characterization of rat and human calpain-5. *Biochem Biophys Res Commun* 324:46-51.
- Wales SQ, Laing JM, Chen L, Aurelian L (2008) ICP10PK inhibits calpain-dependent release of apoptosis-inducing factor and programmed cell death in response to the toxin MPP+. *Gene Ther*.
- Wang H, Yu SW, Koh DW, Lew J, Coombs C, Bowers W, Federoff HJ, Poirier GG, Dawson TM, Dawson VL (2004) Apoptosis-inducing factor substitutes for caspase executioners in NMDA-triggered excitotoxic neuronal death. *J Neurosci* 24:10963-10973.
- Wang KK (1990) Developing selective inhibitors of calpain. *Trends Pharmacol Sci* 11:139-142.
- Wang KK (1999) In: Calpain substrates, assay methods, regulations and its inhibitory agents. In Wang K.K. Yuen P.W. (Eds), *Calpain : Pharmacology and Toxicology of Calcium-Dependent Protease*, pp 77-101: Taylor and Francis, Philadelphia, PA.
- Wang KK (2000) Calpain and caspase: can you tell the difference?, by Kevin K.W. Wang Vol. 23, pp. 20-26. *Trends Neurosci* 23:59.
- Wang KK, Yuen PW (1994) Calpain inhibition: an overview of its therapeutic potential. *Trends Pharmacol Sci* 15:412-419.
- Wang KK, Yuen PW (1997) Development and therapeutic potential of calpain inhibitors. *Adv Pharmacol* 37:117-152.
- Wang KK, Nath R, Posner A, Raser KJ, Buroker-Kilgore M, Hajimohammadreza I, Probert AW, Jr., Marcoux FW, Ye Q, Takano E, Hatanaka M, Maki M, Caner H, Collins JL, Fergus A, Lee KS, Lunney EA, Hays SJ, Yuen P (1996) An alpha-mercaptoacrylic acid derivative is a selective nonpeptide cell-permeable calpain inhibitor and is neuroprotective. *Proc Natl Acad Sci U S A* 93:6687-6692.
- Wang X, Wang F, Sy MS, Ma J (2005) Calpain and other cytosolic proteases can contribute to the degradation of retro-translocated prion protein in the cytosol. *J Biol Chem* 280:317-325.

- Wang X, Yang C, Chai J, Shi Y, Xue D (2002) Mechanisms of AIF-mediated apoptotic DNA degradation in *Caenorhabditis elegans*. *Science* 298:1587-1592.
- Wang Y, Dawson VL, Dawson TM (2009a) Poly(ADP-ribose) signals to mitochondrial AIF: A key event in parthanatos. *Exp Neurol*.
- Wang Y, Kim NS, Li X, Greer PA, Koehler RC, Dawson VL, Dawson TM (2009b) Calpain Activation Is Not Required for Aif Translocation in Parp-1-Dependent Cell Death (Parthanatos). *J Neurochem*.
- Weil ZM, Norman GJ, DeVries AC, Nelson RJ (2008) The injured nervous system: a Darwinian perspective. *Prog Neurobiol* 86:48-59.
- Wendt A, Thompson VF, Goll DE (2004) Interaction of calpastatin with calpain: a review. *Biol Chem* 385:465-472.
- Wingrave JM, Schaecher KE, Sribnick EA, Wilford GG, Ray SK, Hazen-Martin DJ, Hogan EL, Banik NL (2003) Early induction of secondary injury factors causing activation of calpain and mitochondria-mediated neuronal apoptosis following spinal cord injury in rats. *J Neurosci Res* 73:95-104.
- Woodfield K, Ruck A, Brdiczka D, Halestrap AP (1998) Direct demonstration of a specific interaction between cyclophilin-D and the adenine nucleotide translocase confirms their role in the mitochondrial permeability transition. *Biochem J* 336 (Pt 2):287-290.
- Wu M, Yu Z, Fan J, Caron A, Whiteway M, Shen SH (2006) Functional dissection of human protease mu-calpain in cell migration using RNAi. *FEBS Lett* 580:3246-3256.
- Xu L, Deng X (2006a) Suppression of cancer cell migration and invasion by protein phosphatase 2A through dephosphorylation of mu- and m-calpains. *J Biol Chem* 281:35567-35575.
- Xu L, Deng X (2006b) Protein kinase C α promotes nicotine-induced migration and invasion of cancer cells via phosphorylation of micro- and m-calpains. *J Biol Chem* 281:4457-4466.
- Xu W, Yao K, Wang KJ, Wu RY, Sun ZH (2003) [Prevention of oxidative cataract by PD150606 - an inhibitor of calpains]. *Zhonghua Yan Ke Za Zhi* 39:400-405.
- Yamashima T (2000) Implication of cysteine proteases calpain, cathepsin and caspase in ischemic neuronal death of primates. *Prog Neurobiol* 62:273-295.
- Yano Y, Shiba E, Kambayashi J, Sakon M, Kawasaki T, Fujitani K, Kang J, Mori T (1993) The effects of calpeptin (a calpain specific inhibitor) on agonist induced microparticle formation from the platelet plasma membrane. *Thromb Res* 71:385-396.
- Yoshizawa T, Sorimachi H, Tomioka S, Ishiura S, Suzuki K (1995) Calpain dissociates into subunits in the presence of calcium ions. *Biochem Biophys Res Commun* 208:376-383.
- Young W (1993) Secondary injury mechanisms in acute spinal cord injury. *J Emerg Med* 11 Suppl 1:13-22.
- Yu CG, Geddes JW (2007) Sustained calpain inhibition improves locomotor function and tissue sparing following contusive spinal cord injury. *Neurochem Res* 32:2046-2053.
- Yu CG, Joshi A, Geddes JW (2008) Intraspinal MDL28170 microinjection improves functional and pathological outcome following spinal cord injury. *J Neurotrauma* 25:833-840.
- Yu F, Sugawara T, Nishi T, Liu J, Chan PH (2006a) Overexpression of SOD1 in transgenic rats attenuates nuclear translocation of endonuclease G and apoptosis after spinal cord injury. *J Neurotrauma* 23:595-603.

- Yu SW, Andrabi SA, Wang H, Kim NS, Poirier GG, Dawson TM, Dawson VL (2006b) Apoptosis-inducing factor mediates poly(ADP-ribose) (PAR) polymer-induced cell death. *Proc Natl Acad Sci U S A* 103:18314-18319.
- Yu SW, Wang H, Poitras MF, Coombs C, Bowers WJ, Federoff HJ, Poirier GG, Dawson TM, Dawson VL (2002) Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. *Science* 297:259-263.
- Yuste VJ, Moubarak RS, Delettre C, Bras M, Sancho P, Robert N, d'Alayer J, Susin SA (2005) Cysteine protease inhibition prevents mitochondrial apoptosis-inducing factor (AIF) release. *Cell Death Differ* 12:1445-1448.
- Zhang SX, Bondada V, Geddes JW (2003) Evaluation of conditions for calpain inhibition in the rat spinal cord: effective postinjury inhibition with intraspinal MDL28170 microinjection. *J Neurotrauma* 20:59-67.
- Zhang SX, Underwood M, Landfield A, Huang FF, Gison S, Geddes JW (2000) Cytoskeletal disruption following contusion injury to the rat spinal cord. *J Neuropathol Exp Neurol* 59:287-296.
- Zhang W, Lane RD, Mellgren RL (1996) The major calpain isozymes are long-lived proteins. Design of an antisense strategy for calpain depletion in cultured cells. *J Biol Chem* 271:18825-18830.
- Zhang W, Lu Q, Xie ZJ, Mellgren RL (1997) Inhibition of the growth of WI-38 fibroblasts by benzyloxycarbonyl-Leu-Leu-Tyr diazomethyl ketone: evidence that cleavage of p53 by a calpain-like protease is necessary for G1 to S-phase transition. *Oncogene* 14:255-263.
- Zhang X, Chen J, Graham SH, Du L, Kochanek PM, Draviam R, Guo F, Nathaniel PD, Szabo C, Watkins SC, Clark RS (2002) Intranuclear localization of apoptosis-inducing factor (AIF) and large scale DNA fragmentation after traumatic brain injury in rats and in neuronal cultures exposed to peroxynitrite. *J Neurochem* 82:181-191.
- Zhao X, Posmantur R, Kampfl A, Liu SJ, Wang KK, Newcomb JK, Pike BR, Clifton GL, Hayes RL (1998) Subcellular localization and duration of mu-calpain and m-calpain activity after traumatic brain injury in the rat: a casein zymography study. *J Cereb Blood Flow Metab* 18:161-167.
- Zhu C, Wang X, Huang Z, Qiu L, Xu F, Vahsen N, Nilsson M, Eriksson PS, Hagberg H, Culmsee C, Plesnila N, Kroemer G, Blomgren K (2007) Apoptosis-inducing factor is a major contributor to neuronal loss induced by neonatal cerebral hypoxia-ischemia. *Cell Death Differ* 14:775-784.

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MANUSCRIPTS PUBLISHED/ACCEPTED

1. Mitochondrial μ -calpain is not involved in the processing of apoptosis-inducing factor.
Aashish Joshi, Vimala Bondada, and James W. Geddes.
Exp Neurol. 2009 Apr 22 [Epub ahead of print]. PMID: 19393648
2. Intraspinal MDL28170 microinjection improves functional and pathological outcome following spinal cord injury.
Chen-Guang Yu, **Aashish Joshi**, and James W. Geddes.
J Neurotrauma. 2008 Jul;25(7):833-40. PMID: 18627259
3. N-Terminus of calpain 1 is a mitochondrial targeting sequence.
Ramakrishna Badugu, Matthew Garcia, Vimala Bondada, **Aashish Joshi**, and James W. Geddes.
J Biol Chem. 2008 Feb 8;283(6):3409-17. PMID: 18070881

MANUSCRIPTS SUBMITTED/IN PREPERATION

1. RNAi approach reveals a specific role of ERK2 in the deleterious consequences of spinal cord injury.
Chen-Guang Yu, Robert Yezierski, **Aashish Joshi**, Kashif Raza, Yanzhang Li, and James W. Geddes. (*Submitted*)
2. Increased calpain 1 expression during CNS development corresponds with a shift to caspase-independent neuron death.
Yanzhang Li, Vimala bondada, **Aashish Joshi**, and James W. Geddes. (*Submitted*)
3. Calpastatin localization in the mitochondrial intermembrane space.
Aashish Joshi, Vimala Bondada, and James W. Geddes (*In Preparation*)
4. Role of calpain in the processing and distribution of apoptosis-inducing factor.
Aashish Joshi, Vimala Bondada, and James W. Geddes (*In Preparation*)

PUBLISHED ABSTRACTS

1. Intraspinal MDL28170 microinjection improves functional and pathological outcome following spinal cord injury.
Yu CG, **Joshi A**, Geddes JW
Journal of Neurotrauma, Volume: 25, Issue: 7, Pages: 833-840, Published: Jul 2008
2. Targeting ERK2 with lentiviral shRNA improves tissue sparing and locomotor function after spinal cord injury.
Yu CG, Yeziarski RP, **Joshi A**, Kashif Raza, Yanzhang Li, and James W. Geddes
Journal of Neurotrauma, Volume: 25, Issue: 7, Pages: 910-910, Meeting Abstract: P227 Published: Jul 2008
3. The calcium dependent cysteine protease μ -calpain is located in the mitochondrial intermembrane space.
Matthew Garcia, Rama Krishna Badugu, Vimala Bondada, **Aashish Joshi**, and James W Geddes
The FASEB Journal, 2008;22:1017.3.

POSTER PRESENTATIONS

1. Calpastatin localization in the mitochondrial intermembrane space.
Aashish Joshi, Vimala Bondada, and James W. Geddes.
Kentucky Spinal Cord and Head Injury Research Trust symposium, Louisville, KY – 2009.
2. Mitochondrial μ -calpain is not involved in the processing of apoptosis-inducing factor.
Aashish Joshi, Vimala Bondada, and James W. Geddes.
Neuroscience Day, Blue Grass Chapter - Society for Neuroscience, Lexington, KY – 2009.
3. Targeting ERK2 with lentiviral shRNA improves tissue sparing and locomotor function following spinal cord injury in rats.
Cheng-guang Yu, R.P. Yeziarski, **Aashish Joshi**, Kashif Raza, Yanzhang Li, and James W. Geddes.
National Neurotrauma Society meeting, Orlando, FL – 2008.
4. Intraspinal MDL28170 microinjection improves functional and pathological outcome following spinal cord injury.
Cheng-guang Yu, **Aashish Joshi**, and James W. Geddes.
National Neurotrauma Society meeting, Orlando, FL – 2008.
5. Increased calpain 1 expression during CNS development corresponds with a shift to caspase-independent neuron death.
Yanzhang Li, Vimala Bondada, **Aashish Joshi**, and James W. Geddes.
Biology of Calpains in Health and Disease, Snowmass Village, CO – 2007.
6. Is mitochondrial μ -calpain involved in AIF release and caspase-independent cell death?
Aashish Joshi, Vimala Bondada, Mathew Garcia, and James W. Geddes.
Society for Neuroscience 37th annual meeting, San Diego, CA – 2007
7. Inhibition of ERK1/2 by U0126 for spinal cord injury.
Cheng-guang Yu, **Aashish Joshi**, and James W. Geddes.

Society for Neuroscience 37th annual meeting, San Diego, CA – 2007.

8. Mitochondrial μ -calpain: Role in caspase-independent cell death.
Aashish Joshi, Yanzhang Li, Ramakrishna Badugu, Vimala Bondada, Mathew Garcia, Mark Prendergast, and James W. Geddes.
Kentucky Spinal Cord and Head Injury Research Trust Symposium (KSCHIRT), Louisville, KY – 2007.
9. Calpain-1 is located in the mitochondrial intermembrane space.
 Mathew Garcia, Vimala Bondada, **Aashish Joshi**, James W. Geddes.
Society for Neuroscience 36th annual meeting, Atlanta, GA – 2006.
10. μ -calpain is located in the mitochondria.
 Mathew Garcia, Vimala Bondada, **Aashish Joshi**, J.W. Geddes.
Society for Neuroscience 35th annual meeting, Washington DC – 2005.

HONORS AND AWARDS

2009	Kentucky opportunity fellowship (fall semester), University of Kentucky, KY, USA.
2009	Myrle E. and Verle D. Nietzel visiting distinguished faculty program award, University of Kentucky, Lexington, KY, USA.
2008	Selected for attending mitochondrial mini symposium: emergence and convergence, organized by nature genetics and nature neurosciences at Duke University, Durham NC, USA.
2008	Kentucky opportunity fellowship, University of Kentucky, KY, USA.
2007	Graduate school travel award for the society for neuroscience 37 th annual meeting, San Diego, CA - University of Kentucky, Lexington, KY, USA.
2005	Letter of appreciation, from Director of Integrated Biomedical Sciences (IBS) Ph.D. program, University of Kentucky, for excellent academic performance.
2003	Fourth merit position in the faculty of veterinary medicine and animal science, Rajasthan Agriculture University, India.
2000 - 2001	Best cadet of National Service Scheme (NSS), India.
1997	National talent scholarship in agriculture sciences by Indian Council of Agricultural Research (ICAR), New Delhi, India.
1991 - 2003	State Bank of Bikaner and Jaipur (SBBJ) scholarship for excellent academic performance, India.

PROFESSIONAL AFFILIATIONS

2005 - Present	Society for Neuroscience Bluegrass Chapter of the Society for Neuroscience
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TEACHING EXPERIENCE

- | | |
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SERVICE AND LEADERSHIP ACTIVITIES

- Co-organizer of the Department of Anatomy and Neurobiology, University of Kentucky College of Medicine "Translational Neuroscience: Models of Aging 2008 Lexington Conference"- October 2008.
- Served as a Judge thrice in Kentucky State Science & Engineering Fair (KSSEF) held at Eastern Kentucky University, Richmond, KY - March 2009, March 2008 and March 2007.
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